Modulation of intestinal homeostasis and inflammation by *Prevotella intestinalis* (nov. sp.)

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Summary

Intestinal homeostasis is maintained by the dynamic interplay between the gut microbiota and the host immune system. Alterations in the composition and function of the gut microbiota have been associated with a wide range of human disease including inflammatory bowel disease and rheumatoid arthritis. Diverse microbial signatures within the intestinal microbiota have been associated with increased susceptibility to intestinal inflammation, but whether these candidate microbes actively modulate host phenotypes is frequently not known. Metabolites produced through microbiota activity have recently been linked as mediators to distinct intestinal and systemically immune-related disorders.

The role of members of the *Prevotella* genus within the intestinal microbiota and their effects on the host is not completely understood and somewhat conflicting interpretations have been reported. Whereas association with plant-rich diet and improved glucose metabolism suggested *Prevotella* spp. are beneficial for the host, their increased relative abundances in microbial ecosystems at multiple body sites have been associated with diverse diseases. Yet, whether *Prevotella* spp. actively contribute to the development of these diseases is not known. The detailed investigation of the immunomodulatory properties of *Prevotella* spp. has been prohibited by the poor characterization of *Prevotella* species and high strain diversity, as well as the lacking availability of diverse intestinal *Prevotella* isolates.

In the present work, we isolated three novel intestinal *Prevotella* species from mice prone to intestinal inflammation. Characterization of the impact of *Prevotella* colonization on the interplay between host and the microbiota during intestinal homeostasis and inflammation was performed using *P. intestinalis,* which among the three novel species shared the highest similarity to the predominant human gut *Prevotella* species - *P. copri*. We identified that colonization by this novel member of the *Prevotella* genus significantly decreased the production of the bacterial fermentation product SCFAs and the immunomodulatory cytokine IL-18, which was associated with an increase in the severity of intestinal inflammation. Our findings suggested that *Prevotella*-mediated intestinal injury may be influenced via different pathways, yet the ability to ameliorate *Prevotella*-induced disease severity by supplementation of IL-18 suggested that remodeling of the microbial metabolome and specifically SCFA production may be the dominating pathomechanism. Finally, the consequences of modulation of SCFA production in the intestine by *Prevotella* spp. may have far-reaching consequences for the host, as SCFA have immunomodulatory effects in distant sites such as the liver, bones or the brain.

Zusammenfassung

Die intestinale Homöostase wird durch dynamische Wechselwirkungen der Darmmikrobiota und dem Immunsystem aufrecherhalten. Verämderungen bei der Zusammensetzung und Funktion der Mikrobiota wurden mit diversen Krankheitsbildern im Menschen assoziiert, wie entzündliche Darmerkrankungen und rheumatoider Arthritis. Auch wenn diverse mikrobielle Signaturen mit einer erhöhten Anfälligkeit für Darmentzündungen assoziiert werden konnten, bleiben die ursächlichen Mikroben, welche die auftretenden Phenotypen aktiv modulieren, unbekannt. Kürzlich konnten von der Mikrobiota produzierte Metabolite als Mediatoren für Darmentzündungen und systemische Immunerkrankungen identifiziert werden.

Die Rolle von Bakterien aus dem *Prevotella* Genus und ihre Effekte auf den Wirt im Kontext von Darmentzündungen führte zu gegensätzliche Interpretationen. Zum einen wurde eine erhöhte Anzahl an *Prevotella* spp. mit einer pflanzenreichen Ernährung und einem verbesserten Glucose-Metabolismus assoziiert, zum anderen wurde *Prevotella* spp. in mikrobiellen Gemeinschaften mit diversen Krankheiten in Verbindung gebracht. Ob *Prevotella* spp. aktiv bei der Entstehung dieser Krankheiten beiträgt ist nicht bekannt. Eine detaillierte Untersuchung der immunmodulatorischen Eigenschaften von *Prevotella* spp. wurde bisher durch mangelnde Charakterisierungen der *Prevotella*-Spezies, die große Vielfalt der bekannten Stränge und die fehlende Verfügbarkeit verschiedener *Prevotella*-Isolate des Darmes erschwert.

In dieser Arbeit wurden drei neue intestinale *Prevotella* Spezies aus Mäusen isoliert, die anfällig für intestinale Entzündungen sind. Die Rolle von *Prevotella* beim Wechselspiel zwischen Wirt und der Mikrobiota bei intestinaler Homöostase und Entzündung wurde mit dem Isolat *P.intestinalis* untersucht, welches von den neu isolierten Spezies die höchste Ähnlichkeit zu der im humanen Darm dominanten *Prevotella* Spezies (*P. copri*) aufweist. Es konnte gezeigt werden, dass eine Besiedlung mit dieser *P. intestinalis* sowohl die bakterielle Produktion von kurzkettigen Fettsäuren (SCFAs) als auch die Bildung des immunmodulatorischen Zytokins IL-18 signifikant verringert, was wiederrum mit einem erhöhten Schweregrad einer intestinalen Entzündung assoziiert wird. Dies impliziert, dass die durch *Prevotella* mediierte intestinale Enzündung durch unterschiedliche Signalwegen beeinflusst werden kann. Da die *Prevotella*-induzierte Krankheitsschwere durch eine Supplementierung von IL-18 gelindert werden kann, stellt die Veränderung des mikrobiellen Metaboloms und vor allem der Bildung der SCFAs möglicherweise den dominierenden Pathomechanismus dar. Da SCFAs immunmodulatorische Effekte an unterschiedlichsten Stellen des Körpers wie Leber, Knochen oder Gehirn haben, hat die Modulierung der SCFA-Produktion im Darm durch *Prevotella* spp. damit letztendlich vermutlich weitreichende Folgen für den Wirt.

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Abbreviations

AIEC	adherent-invasive *E. coli*
AIM2	absent in melanoma 2
ALRs	AIM2-like receptors
AMPs	antimicrobial peptides
APCs	antigen-presenting cells
ATG16L1	autophagy related 16-like 1
ATP	adenosine triphosphate
BMDM	Bone Marrow-derived Macrophages
BSA	bovine serum albumin
CD	Crohn's disease
CEC	cecum
DAMPs	damage-associated molecular patterns
DC	distal colon
DCs	dendritic cells
DNA	deoxyribonucleic acid
DSS	dextran sodium sulfate
ELISA	Enzyme-linked immunosorbent assays
ETBF	enterotoxigenic *B. fragilis*
FBS	fetal bovine serum
FFRs	free fatty acid receptors
Foxp3	forkhead box P3
GF	germ-free
GPR	G-protein-coupled receptor
GWAS	genome-wide association studies
HDACs	histone deacetylases
HRP	horse radish peroxidase
HZI	Helmholtz Centre for Infection Research
IBD	inflammatory bowel disease
IECs	intestinal epithelial cells
IFN	interferon
IgA	immunoglobulin A
IL	interleukin
IRGM	immunity-related GTPase M protein
IVCs	individually ventilated cages
LDA	linear discriminant analysis
LPLs	lamina propria lymphocytes
LPS	lipopolysaccharide
MAMPs	microbe-associated molecular patterns
MDP	muramyl dipeptide
MLN	mesenteric lymph nodes
MyD88	myeloid differentiation primary response protein 88

NCI	National Cancer Institute
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NGS	next generation sequencing
NLRP	NLR family, pyrin domain containing
NLRs	nucleotide-binding domain and leucine-rich repeat-containing receptors
NOD2	nucleotide oligomerization domain 2
PC	proximal colon
PCoA	principle coordinates analysis
PCR	polymerase chain reaction
PRRs	pattern recognition receptors
PSA	polysaccharide A
PUL	polysaccharide utilization loci
Rag	Recombination Activating 2
RELM	resistin-like molecule
RELMβ	resistin-like molecule beta
RIG-I	retinoic acid-inducible gene I
RLR	RIG-I like receptors
RNA	ribonucleic acid
SAA	serum amyloid A
SCFAs	short-chain fatty acids
SDS	sodium dodecyl sulfate
SFB	segmented filamentous bacteria
SI	small intestine
SPF	specific pathogen free
TJ	tight junctions
TLRs	Toll-like receptors
TNBS	trinitrobenzene sulfonic acid
TNF-α	tumor necrosis factor - α
Treg	regulatory T cell
UC	ulcerative colitis
WT	wild type

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Introduction

1.1 Inflammatory bowel disease

Inflammatory bowel disease (IBD) comprises a group of intestinal disorders that cause prolonged inflammation of the digestive tract. Most cases of IBD are classified as either ulcerative colitis (UC) or Crohn's disease (CD). While ulcerative colitis generally affects the innermost lining of the intestinal wall and is characterized by continuous inflammation of the colon and rectum, Crohn's disease can manifest as ulcerations and damage of all layers of the intestinal wall of any part of the gastrointestinal tract[1]. IBD was once a very rare disorder, and its incidence only began to rise in the second half of the 20th century. In time-trend analyses, 75% of CD studies and 60% of UC studies showed increased incidence rates over times[2] with estimated 53,000 new cases of CD and 123,000 new cases of UC each year in Europe[3]. The highest reported prevalence of IBD is in Europe (UC, 505 per 100,000 persons; CD, 322 per 100,000 persons) and North America (UC, 249 per 100,000 persons; CD, 319 per 100,000 persons. Cases are much less common in non-Western nations, although IBD has emerged in newly industrialized countries in Asia, South America and the Middle East and has evolved into a global disease with rising prevalence in every continent[4].

Although the etiology of IBD in a classic sense remains unknown, multiple hereditary, environmental, and lifestyle factors are thought to influence the pathology of the disease. It is now widely recognized that the cause of IBD originates in an extremely complex interaction of aberrant immune responses to members of intestinal microbes, further complicated by genetic and environmental factors[5]. Importantly, none of these factors is

likely to cause the disease alone and the ongoing research aims to solve how these factors individually or collectively contribute of the disease development.

1.1.1 Genetic factors in IBD

The fastest progress in understanding the IBD pathogenesis has been done in the field of genetic studies. Genome-wide association studies (GWAS) have revealed over 230 IBD susceptibility loci, substantially more than reported for any other complex disease[6]. Some of the most prominent genetic associations to IBD are the genomic regions containing nucleotide oligomerization domain 2 (NOD2), autophagy-related genes, multiple genes involved in T helper (Th) 17 cells and interleukin (IL)-23 signaling pathway as well as variants in *IL10* loci. NOD2, an intracellular sensor which stimulates an immune reaction upon microbial recognition, was the first identified CD susceptibility gene[7,8]. Genetic analyses in IBD patients reported single nucleotide polymorphisms (SNPs) in two genes involved in autophagy, autophagy related 16-like 1 (*ATG16L1*), and immunity-related GTPase M protein (*IRGM*)[9–12]. Autophagy not only clears intracellular components but also removes invading intracellular bacteria. Furthermore, a significant association between IBD and the *IL23R* gene has been described[13]. The *IL23R* gene encodes a subunit of the receptor for the proinflammatory cytokine IL-23, a molecule involved in the generation of Th17 cells. Among identified susceptibility loci for IBD, there are also genes related to the inflammasome components like members of the nucleotide binding domain and leucine-rich repeat-containing proteins NLR family, i.e. NLR family pyrin domain containing 3 (NLRP3), including downstream related protein IL-18[14,15]. Recently, another NLR protein, NLRP6, has been found to be highly expressed in colon biopsies from active ileal CD patients [16]. Both NLR proteins are intracellular receptors that are directly or indirectly activated by microbial products and have been shown to play an important role in intestinal inflammation in mouse models[17–19].

Interestingly, not all genetic susceptibilities present the same risk across the world. It has been noted that, for example, *ATG16L1* gene represents a higher risk for CD in the population of European descent, but not in Asian people. Notably, in Asia only 3% of people with IBD have a close relative with the disease, compared with 15% of individuals in the Western world[20]. Furthermore, studies in monozygotic twins showed that the concordance rate for UC is 10–15% compared with 30–35% in CD[21,22]. These observations suggest that genetic factors are responsible for only a part of the overall disease variance. In addition,

most of the evidence related to a possible genetic cause point to pathways essential for intestinal homeostasis, innate and adaptive immune regulation and microbial defense. These elements support the hypothesis that microbial interactions with the immune system play a key role in IBD. The interplay between intestinal microbes and mucosal immune system in the intestine is the focus of the present work and is reviewed in more details below.

1.1.2 Environmental triggers in IBD

Multiple environmental factors have been found to contribute to the development of IBD[23]. Knowing that the prevalence of IBD has been changing in the last 20 years and that the incidence is on the rise in developing countries, westernization of lifestyle and industrialization have been implicated as major factors[24]. This includes changes in hygiene, diet, and medical treatments. Among medical treatments, oral contraceptives, post-menopausal hormone replacement, aspirin, NSAIDs, and antibiotics have been implicated in increased risk of CD or UC[25]. Most of these factors are known to influence the composition of the intestinal microbial communities, making it unclear whether they are a primary or secondary event[26].

One of the most studied environmental triggers for CD and UC is smoking. Several studies showed that smoking increases the risk of developing CD by two fold, while it has protective effects on UC. Regardless of strong epidemiological data, the reasons for the opposite effects of smoking on CD and UC remain unclear and require further investigation[25,27,28].

1.2 The intestinal epithelial barrier

It has been hypothesized that IBD arises from dysregulated immune responses towards the intestinal microbial communities[1,29]. To prevent aberrant immune response, intestinal epithelial cells serve as a physical and biochemical barrier that separates the host tissue and commensal bacteria necessary to maintain the intestinal homeostasis. Therefore, impairment of the barrier functions is thought to promote intestinal inflammation IBD[30].

Being exposed to numerous antigens from potentially harmful microbes but also harmless dietary components, the intestinal mucosal immune system evolved a wide range of defense

strategies to monitor the gastrointestinal tract for the presence of pathogens, while tolerating trillions of commensal bacteria. In the last two decades, it became evident that the microbial communities in the intestine are not just a bystander, but they play a fundamental role in the induction, education, and function of the mucosal but also systemic host immune system[31]. This continuous interaction starts already in the womb, intensifies after birth and is dynamic throughout life thereby continuously shaping the immune response[32]. The biggest contribution to our knowledge about the importance of the microbiota in shaping host immunity comes from germ-free (GF) models, as they display an 'underdeveloped' innate and adaptive immune system: reduced expression of antimicrobial peptides, reduced IgA production, reduced T cell diversification and recruitment resulting in increased susceptibility to microbial infections[33–36]. Conversely, the host immune system influences the microbiota composition through various mechanisms, as seen in several immune deficiencies[37–39]. These strong interconnections between the microbiota and the intestinal immune system make studying one without the other rather difficult. In general, these findings demonstrate that the microbiota and the host immune system have a complex, dynamic, and reciprocal dialogue.

The single layer of intestinal epithelium plays a central role in maintaining the homeostasis in the intestine. It positions itself as a barrier between the vast number of microbial species and their products and the mucosal immune system, thus minimizing their contact and promoting the symbiotic relationship. However, intestinal epithelial cells (IECs) are more than just a physical barrier. Besides their importance in nutrient absorption, IECs preform signal-transduction functions by sensing bacterial products through innate immune receptors and directing an appropriate immune response in the lamina propria (LP). An immune response is triggered as a reaction to numerous endogenous damage-associated molecular patterns (DAMPs) and microbe-associated molecular patterns (MAMPs), which include ligands from non-pathogenic microbes of the microbiota as well[40]. MAMPs are recognized by a range of pattern recognition receptors (PRRs) expressed in IECs and wide range of immune cells, such as Toll-like receptors (TLRs), nucleotide-binding domain and leucine-rich repeat containing receptors (NLRs), retinoic acid-inducible gene (RIG) -I like receptors (RLRs)[41].

Stem cells within the crypt of the intestinal epithelium generate multiple IEC lineages with distinct functions, including absorptive enterocytes (small intestine) and colonocytes (colon), enteroendocrine cell, secretory goblet cells and Paneth cell[42]. Goblet cells form a physical and chemical barrier by producing a two-tiered mucus layer that covers the intestinal

epithelium and restricts bacterial penetration. While the inner layer is densely packed, firmly adherent to the epithelium and devoid of bacteria, the outer layer is more loosely attached and it provides a niche for luminal bacteria that use oligosaccharides from mucin as an energy source[43]. Goblet cells can also produce antimicrobial peptides (AMPs). Paneth cells are located in the crypts of the small intestine and are contributing to the innate immune response by secreting AMPs in reaction to resident microbes or invading pathogen in the lumen. The AMPs produced in the intestines include the β-defensins and cathelicidins produced by colonocytes, α-defensins, lysozyme, phospholipase A2, and RNases produced by Paneth cells, while C-type lectins are produced by most IEC lineages[44]. Some of these AMPs, such as α-defensins, are expressed constitutively, while others are secreted upon sensing of distinct MAMPs, frequently derived or also present in commensal bacteria. For instance, it has been shown that lectins, such as regenerating islet-derived protein (Reg)IIIγ, RegIIIβ, and resistin-like molecule (RELM) β, are induced by microbial signals in a TLR-MyD88 (myeloid differentiation primary response protein 88)-dependent manner[45]. Another study showed that intestinal AMP expression, specifically *Retnlb, Ang4,* and *Itln1*, is regulated by inflammasome-derived IL-18[46]. Under homeostatic conditions, IEC represent a major source IL-18[47] and several studies have suggested IL-18 is a key mediator of intestinal homeostasis and inflammation. However, seemingly opposing results have been reported, suggesting both beneficial and detrimental properties of IL-18 in the intestine[48,49]. Most recent reports will be discussed in detail in the following section.

Taken together, the intestinal epithelium represents not only a physical and chemical barrier but as well is an important mediator of the dialog between the intestinal microbes and the host immune system by the production of proinflammatory mediators, which regulate the immune responses and promote the maintenance of intestinal homeostasis.

1.2.1 Role of interleukin-18 in the intestine: friend or foe?

Interleukin-18 (IL-18) is a member of the IL-1 family of cytokines[50]. Similar to IL-1β, IL-18 is initially synthesized intracellularly as an inactive precursor where it remains[51]. Processing of the IL-18 precursor into an active mature form is performed by the intracellular cysteine protease caspase-1. Comparable with the processing of IL-1β, autocatalytic cleavage of inactive pro-caspase-1 first generates active caspase-1 upon assembly of distinct inflammasome complexes, including nucleotide-binding domain and leucine-rich repeat pyrin containing proteins (NLRPs) and absent in melanoma 2-like receptors (ALRs, AIM2-

like receptors) [52]. Despite similarities, the biology of IL-1β and IL-18 is diverse. Interestingly, while gene expression of IL-1β in hematopoietic cells is absent in during steady state (healthy humans and mouse), the IL-18 precursor is constitutively present in nearly all epithelial cells and blood monocytes[51,53]. IEC have been shown to represent a major source of IL-18 in mice[47].

In the last two decades, numerous studies have shown that IL-18 plays an import role in intestinal inflammation, however seemingly opposing results have been reported. Genome-wide association studies have revealed a number of polymorphisms associated with disease susceptibility, including the association of mutations within the IL18R1-IL18RAP locus with both adult and severe early-onset IBD[54,55]. Furthermore, intestinal biopsies from IBD patients were characterized by increased concentrations of IL-18 produced by both IECs and macrophages[56]. In addition, a number of studies have used IL-18-deficient mice or neutralization of endogenous IL-18 and demonstrated its pro-inflammatory role in driving the intestinal inflammation[57,58]. These data suggested that neutralization of IL-18 would have beneficial effects in ameliorating the inflammation. However, data associating polymorphisms in the NLRP3 region with increased susceptibility to CD questioned this hypothesis[15]. Interestingly, functional studies in mice demonstrated that the deletion of the NLRP3 inflammasome, which consequently resulted in decreases of IL-18 and IL-1β, increased the susceptibility to intestinal inflammation[19]. The same study showed that administration of IL-18 could reverse the disease.

In the past years, the influence of alterations with the microbiota composition on disease modulation and phenotypic discrepancies have been observed in numerous experimental animal models[59,60]. Specifically, several studies have addressed in the recent years the role of IL-18 in the intestine considering the influence of the microbiota and excluding its effects by microbiota normalization. It has been shown that caspase-1 and IL-18 exacerbate intestinal inflammation due to impaired repair processes[61,62]. In contrast, it has been demonstrated that deficiency of NLRP6 results in reduced IL-18 levels and microbial dysbiosis, which consequently leads to increased susceptibility to intestinal inflammation[18]. Microbial dysbiosis was suggested to arise from decreased IL-18 production and AMP levels, which are modulated by microbiota-associated metabolites taurine and distinct polyamines[46]. Several of these studies showed that supplementation of IL-18 ameliorates the intestinal inflammation in the dextran sodium sulfate (DSS) colitis model. Interestingly, recent work showed that also short-chain fatty acids (SCFA) have the capacity to alter the

levels of IL-18 production[63]. They suggested that IL-18 production, stimulated by SCFA, promotes epithelial barrier integrity and regeneration.

To explain these differences it has been proposed that IL-18, since constitutively expressed in the intestinal epithelium, has a protective role by contributing to the maintenance of the intestinal barrier by enhancing the regeneration of the damaged epithelium. After damage to the barrier, the microbial products stimulate macrophages in the lamina propria and excessive caspase-1 dependent processing of IL-18 results in inflammation, suggesting the cellular source might be of crucial importance for the observed opposing effects[49]. However, this has not yet been proven experimentally. Moreover, the earlier analysis suggested IL-18 is cytoprotective in the early stages of IBD, whereas chronic production exacerbates the disease[64]. In addition, it has been discussed that IL-18 ameliorates the inflammation in innate colitis induced by intestinal injury (i.e. DSS), whereas it exacerbates the disease in T-cell dependent colitis i.e. 2,4,6-trinitrobenzene sulfonic acid (TNBS) by promoting IFN-γ production in T cells[65,66]. However, another potential explanation may be that the composition of the gut microbiota determines which immune pathway is activated and the susceptibility to the diseases, not only the model of colitis used[67].

In summary, despite great efforts to understand the role of IL-18 in intestinal inflammation, its particular role in IBD still remains controversial. Understanding the complex interplay between the IECs, microbiota and their metabolites, and the immune system that defines the balance of IL-18 in the intestine might be crucial for differentiating its protective and detrimental effects on the host.

1.3 Intestinal immune defense

Along with AMPs, secretion of immunoglobulin (Ig) A by plasma cells is additionally reinforcing the intestinal barrier against invading microbes. Gut plasma cells that produce IgA can be generated in T cell-dependent and T cell-independent mechanisms. While enteric pathogens have been known to induce high affinity T cell-dependent, pathogen-specific IgA, commensals induce IgA of low affinity and specificity[68,69]. Some commensal bacteria, such as segmented filamentous bacteria (SFB) and *Mucispirillum* spp., that colonize the inner mucus layer induce high-affinity T cell-dependent IgA response[69,70]. It has been suggested that intestinal bacteria that are highly coated with IgA have the potential to promote inflammation. Palm et al. showed that colitogenic intestinal communities display an

increase in the percentage of IgA-coated (IgA+) bacteria. Sequencing of these bacteria found Prevotellaceae, Helicobacteraceae, and segmented filamentous bacteria (SFB) to be among the highly coated ones[69]. Moreover, in the same study transferring the IgA+ bacteria from IBD patients to mice exacerbated intestinal inflammation in the animal model of IBD.

The intestinal barrier is additionally complemented by cells of innate and adaptive immune system. Myeloid antigen-presenting cells (APCs) of the intestine are a heterogeneous population consisting of dendritic cells (DCs) and macrophages. Like in most tissues, intestinal macrophages have essential phagocytic and bactericidal activities that help to maintain the intestinal homeostasis. Located in the proximity to high numbers of luminal bacteria and antigens, macrophages are a part of first-line defense when the epithelium barrier fails due to an infection or an injury[71]. As the intestinal macrophages are unable to proliferate, they are derived from circulating monocytes, which are attracted in steady state and during inflammation to the mucosa, where they further differentiate. It has been shown that after differentiation in the steady state, resident macrophages have downregulated expression of innate receptors for lipopolysaccharide (LPS) - CD14 and IgA and decrease the production of pro-inflammatory cytokines[72]. Hyporesponsiveness of intestinal macrophages has been linked to specific microbial metabolites, namely SCFAs[73]. Such regulation of immune responses has an important function in promoting tolerance and preventing aberrant inflammatory responses.

Intestinal dendritic cells are the central player monitoring the environment, responding to distinct stimuli, and linking the innate and adaptive immune responses. DC sample antigen from transcytosed bacteria in the Payer´s patches or directly from the lumen and present them to naive T cells in mesenteric lymph nodes (MLN)[74]. Whether the DCs will mount a tolerogenic or inflammatory response depends on epithelial-derived factors and bacteria-derived products. These factors together determine the fate of naive CD4+ T helper (Th) cells during priming in MLN[75].

$CD4^+$ T cells are a principal component of the adaptive immune system. In the intestine, $CD4^+$ T cells are mostly located in the lamina propria. Once stimulated, naive $CD4^+$ T cells can differentiate into four major subtypes: T helper 1 (Th1), Th2, Th17, or regulatory T cell (Treg), which can be distinguished based on their expression of various transcription factors and cytokines. For example, Th1 cells are critical for the host defense against intracellular microbial infection, while Th2 cells play an important role in eliminating parasite infections. Regulation and balance between the CD4+ T cells subtypes have been shown to be important for maintenance of the intestinal homeostasis. Uncontrolled Th responses can be

pathological, as the Th1 and Th17 responses have been linked to autoimmune diseases, while the Th2 response has been associated with allergic reactions[76]. In contrast, Tregs promote anti-inflammatory response and are a key mediator of immune tolerance. Dysfunction of Tregs can lead to exacerbated inflammation and autoimmune disorders[77].
Interestingly, individual members of intestinal microbial communities have been found to shape different aspects of immune responses and their role and examples are discussed in more detail in section “1.5 Intestinal Commensals as Immune Modulators”.

1.4 The intestinal microbial community

The microbial communities of the intestinal tract of vertebrates are composed of bacteria, viruses, eukaryotes, archaea, and fungi, which are collectively referred to as the “microbiota”. Distinct microbial communities can colonize all epithelial surfaces, but the intestinal bacterial members represent the densest and metabolically active community[78]. Recent large studies characterizing microbial communities that inhabit the human body estimated that over 1,000 bacterial species can be found in the intestine, of which the most abundant are Firmicutes, Bacteroidetes, and Actinobacteria, whereas Proteobacteria, Fusobacteria, Cyanobacteria, and Verrucomicrobia are less represented[78,79]. The microbiota composition in every individual is influenced partly by the host genotype and physiology, and predominantly by distinct environmental factors, including dietary habits and medications (e.g., antibiotics)[80,81]. Even though studies noted that there is a substantial inter-individual variability in the gut microbiota composition in healthy individuals, metabolic functionality is rather conserved between groups of studied individuals because many biochemical pathways are redundant between alternative members of the microbiome.
For many years bacteria have been classified either as a “commensal” or a “pathogen”, depending on their relationship with the host. However, even though members of the microbiota are often referred to as commensals, the form of the crosstalk can vary. Many of the microbes found in the gut coevolved with the host and developed beneficial mutualistic relationships (Mutualist), while others live in association with a host without obvious benefit or harm to either member (Symbiont). Interestingly, there are also members of indigenous microbiota that usually do not cause a disease but can exploit disrupted homeostasis and exert pathogenic effects on the host. For these microorganisms, the term pathobiont has been proposed[31,74]. Specifically, the term “pathobionts” has been coined to distinguish them

from classical acquired opportunistic pathogens. Additionally, commensal bacteria that have immunomodulatory properties impacting the host´s immune system, but are not associated with a disease, have been referred to as “autobionts”[82]. Clearly, the terminology used to describe the members of the intestinal microbiota based on their relationship with the host and functional properties is still evolving.

It has been by now widely acknowledged that the gut microbiota confers many benefits to the host by performing a wide range of metabolic functions. These include food digestion, bioconversion of nutrients, metabolism of drugs and xenobiotics, protection against pathogens, and regulation of host immunity[83–85]. The interplay between the microbiota and the immune system is crucial for maintenance of regulatory pathways involved in the maintenance of intestinal homeostasis. Moreover, it has been hypothesized that change of lifestyle in developed countries, overuse of antibiotics, changes in diet, and a decrease in exposure to diverse microorganisms and parasites select a microbiota that lacks the resilience and diversity required for immune education and stimulation of immune-regulatory responses[59]. This might contribute to the uneven geographical distribution of inflammatory and autoimmune diseases in the world[4].

1.4.1 Microbial dysbiosis and IBD

A decade of genome-wide association studies and genetic studies has highlighted the importance of the host–microbe interactions in the pathogenesis of IBD and proposed a link between the aberrant immune response in IBD patients to the intestinal microbiota. In the recent years, intestinal microbiome studies have associated compositional and functional shifts of the intestinal microbiome, also known as dysbiosis, to pathogenesis of many infectious and inflammatory diseases[31,86]. Dysbiosis can occur as a result of the loss of beneficial microorganisms, the expansion of potentially harmful microorganisms or pathobionts, or loss of overall diversity[87]. The same factors that influence the composition of the microbiota can contribute to the development of the dysbiosis, such as diet, enteric infections, use of medications, hygiene, physical activity, familial transmission, and genetics[88].

In general, an overall loss of microbial diversity, dysbiosis, and changes in microbiota metabolic output have been reported in IBD patients and are hypothesized to promote disease development[89–91]. The exact identity of specific bacterial members that can trigger

aberrant host responses and contribute to IBD development in humans is not exactly known since direct causal relationships between microbiota and IBD have been difficult to prove outside animal models. Nevertheless, several studies in humans described associations between IBD and increased abundance in Gammaproteobacteria and presence of Enterobacteriaceae, particularly adherent-invasive *E. coli* (AIEC) strains[92]. Another study analyzed the microbiota of a large cohort of newly diagnosed, treatment-naive children with CD, and demonstrated an increased abundance of Enterobacteriaceae, Pasteurellaceae, Veillonellaceae, and Fusobacteriaceae in ileal and rectal biopsy samples[93]. In mouse models, additional members of the microbiota were identified to directly exacerbate intestinal inflammation or were found to be enriched in communities which promote disease severity. This includes members of the Enterobacteriaceae family, i.e. *Klebsiella pneumoniae* and *Proteus mirabilis*[94,95], *Akkermansia muciniphila*[96], distinct *Bacteroides*[97] and *Helicobacter* species[98], as well as members of *Prevotella* genus[18,99]. Currently, it remains unknown whether dysbiosis of the gut microbiota is the cause of inflammation or a result of bacterial adaptation to the new intestinal environment caused by the inflammation.

1.5 Intestinal commensals as immune modulators

In the recent years progress has been made in understanding the interactions between the microbiota and the mucosal immune system. As the influence of the microbiota on regulation of immune functions becomes evident, characterizing the role of individual commensal bacteria became an area of scientific and clinical relevance. With the advancement of “next generation sequencing” (NGS) methods and utilization of gene-deficient and gnotobiotic animal models, microbial communities and individual commensals have now been demonstrated to specifically modulate different aspects of host immunity. Remarkably, gut microbiota has a role beyond the local gut immune system and impacts many systemic immune components. For instance, modulation of immune responses may result in alteration of enteric infection susceptibility as well as development of autoimmune-related diseases[100,101]. The following sections give a brief overview of several well-studied examples of specific members of the gut microbiota that have been linked with immunomodulatory properties.

1.5.1 *Bacteroides fragilis*

Among the numerous *Bacteroides* species that have been reported to modulate host immune responses, *B. fragilis* is the most studied one. Interestingly, both anti- and pro-inflammatory properties have been reported for distinct strains of *B. fragilis*. The first report showing a unique commensal molecule could promote regulatory responses was demonstrated by the identification of the polysaccharide A (PSA) produced by a *B. fragilis* strain[102]. Presence of polysaccharide A in *B. fragilis* is responsible for expansion of Tregs via TLR2 signaling in germ-free mice[103]. Induction of such immune-regulatory responses provides protection from chemically-induced colitis in animal models. Furthermore, PSA in *B. fragilis* was shown to promote IL-10 secretion in CD4+ cells, while suppressing Th17 pro-inflammatory response[104]. Another strain, enterotoxigenic *B. fragilis* (ETBF) has been linked with potent Th17 response and severe colitis[105].

1.3.2 *Clostridium* spp.

Clostridia are a heterogeneous group of Gram-positive, spore-forming bacteria within the Firmicutes phylum including a large number of species of commensal bacteria and human pathogens, such as toxin-producing members *Clostridium tetani, C. perfringens, and C. difficile*. Commensal non-toxicogenic Clostridia have been implicated in the maintenance of mucosal homeostasis and prevention of IBD[106]. Induction of Treg cells is not restricted to *B. fragilis* as the presence of an indigenous Clostridium species also promotes Treg cell expansion by promoting TGF-β secretion[107]. Interestingly, induction of Tregs in the colon was increased when mice were colonized with a consortium of *Clostridia* spp., whereas individual species had a modest effect on the immune system[108]. Clusters IV and XIVa were demonstrated to play a significant role in maintaining intestinal function by producing butyrate[109]. Furthermore, independent studies reported these two Clostridia clusters to be reduced in IBD patients[106,110].

1.5.3 *Faecalibacterium prausnitzii*

F. prausnitzii, a member of the Clostridia, is one of the most prevalent species in the human gut microbiota[111,112]. It has been associated with health benefits for the host due its

production of SCFA, mostly butyrate. Butyrate has been linked to anti-inflammatory effects as its presence modulates expansion of regulatory Tregs and secretion of IL-10 in dendritic cells[113]. Importantly, *F. prausnitzii* has been routinely found in lower relative abundances in UC patients compared to healthy individuals[114,115]. In animal models it has been further demonstrated that administration of *F. prausnitzii* leads to a significant decrease in inflammation severity[116]. Moreover, it has been demonstrated that *F. prausnitzii* ameliorates colorectal colitis by inhibition of IL-17 in rats[117]. Additional mechanisms of anti-inflammatory effects in *F. prausnitzii* have been reported, such as the inhibitory effect on NF-kB signaling in IECs *in vitro*[110].

1.5.4 Segmented filamentous bacteria (SFB)

SFB are Gram-positive, spore-forming, filamentous bacteria reaching up to 80 µm in length[118]. Analysis of 16S rRNA sequence of mouse SFB revealed that the microbes belong to a novel genus in the order *Clostridiales*[119]. Although initially discovered in murine intestine[118], there is now evidence that SFB-like bacteria exist in a broad range of species, including humans, non-human primates, chickens, horses, and other animals[120,121]. The first segment of the microbe possesses a nipple-like appendage that projects into the plasma membrane of the enterocyte, without actually rupturing or penetrating the host cell wall[118]. This close contact with IECs allows them to exert strong immunomodulatory properties. They are best known for their role in expansion of Th17 cell in small intestine which has been shown to provide protection in the large intestine against enteric *Citrobacter rodentium* infection[122]. However, recruitment of SFB-induced Th17 cells to systemic sites can have detrimental effects by promoting inflammatory disease, such as arthritis and multiple sclerosis[123]. SFB are also known to induce serum amyloid protein A (SAA), which via dendritic cells impacts the IL-22 production in innate lymphoid cells (ILCs)[124]. Even though SFB induce Th17 cells, they do not cause aberrant intestinal inflammation suggesting Th17 cells induced by SFB may be qualitatively different from pathogenic Th17 induced during colitis or other inflammatory disorders[125].

1.5.5 *Helicobacter* spp.

Helicobacter spp. are Gram-negative, flagellated bacteria within the phylum of Proteobacteria. They are widely present in experimental mouse colonies around the world[126] as well as wild house mice[127]. Numerous species isolated from the intestine of mice are suspected to be pathobionts based on their ability to induce or enhance colitis in immune-deficient mice, but not WT[98,128,129]. Specifically, *H. hepaticus* has been shown to promote intestinal inflammation in Rag2- and IL-10-deficient mice by activation of IL-17 and IFN-γ producing CD4+ T cells[130]. However, infection of WT mice results in the induction of IL-10-producing Treg cells that prevented bacteria-induced colitis[131]. These findings suggested *Helicobacter* in the intestine induce regulatory T cells during homeostasis and effector T cells during colonic inflammation. Besides *H. hepaticus,* other species have been shown to modulate the immune response, including *H. typhlonius* and *H. rodentium*[132]*.*

1.5.6 *Escherichia coli*

E. coli are Gram-negative bacteria and belong to the family of Enterobacteriaceae that includes many commensals, opportunistic bacteria, and strict pathogens[133]. In the last decades, *E. coli* and in particular adherent-invasive *E. coli* (AIEC) has been implicated in the pathogenesis of IBD both in mouse models and IBD patients. AIEC ability to adhere and invade epithelial cells has been shown to trigger TNF-α production in macrophages. The ability of AIEC to adhere and invade epithelial cells triggers TNF-α production in macrophages. Additionally, non-AIEC *E. coli* have been reported to induce Th17 response in GF mice and have the ability to promote inflammation in genetically susceptible host[134]. Immuno-modulatory capability was shown as well in probiotic strain *E. coli* Nissle 1917, which promotes immune regulation by expansion of plasmacytoid DC and Foxp3+Rorgt+CD4+.

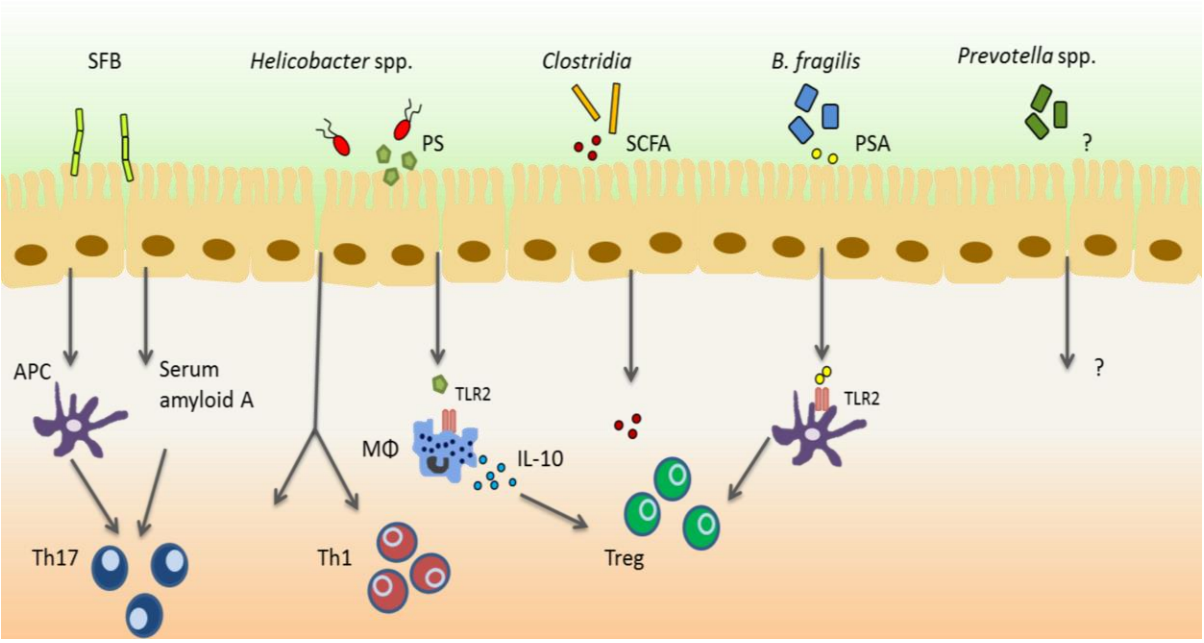


Figure 1: Multiple bacterial species and microbial metabolites modulate immune response in the intestine.

Segmented filamentous bacteria (SFB) induce T helper (Th) 17 cells in the intestinal lamina propria via mechanisms that involve host production of serum amyloid A (SAA) or direct activation via antigen presenting cells (APC). *Helicobacter* species can induce effector T cells and promote inflammation in immuno-deficient host, whereas the *Helicobacter*-derived polysaccharides (PS) have been shown to interact with TLR2 receptor on macrophages (MΦ) promoting IL-10 production and expansion of regulatory T cells (Treg). A group of Clostridia species, taken together or individually, have been demonstrated to induce colonic Treg via production of short-chain fatty acids (SCFAs). Induction of Treg has as well been linked to polysaccharide A (PSA) in *B. fragilis* which interacts with TLR2 on APCs. Although associated to several inflammatory conditions, mechanism of immune modulation by *Prevotella* spp. has not been fully understood.

1.5.7 *Proteus mirabilis*

Other members of Enterobacteriaceae have been reported to play a causative role in intestinal inflammation. Colonization of TRUC (*Tbet*$^{-/-}$*Rag2*$^{-/-}$) mice with both *P. mirabilis* and *K. pneumoniae* induces colitis in specific pathogen free (SPF) mice, but not in GF animals, suggesting their colitogenic properties depend on the microbiota community, rather than the host genetics[94]. Intestinal inflammation in *Proteus*-colonized mice was been later

demonstrated to be mediated by bacterial hemolysin and its ability to induce NLRP3 signaling in monocytes resulting in the production of mature IL-1β[95].

It is clear that the efforts of identifying individual members of the microbiota and their effects on the immune system are still in their infancy. However, scientists have started to transit from describing the association between microbial communities and diseases to investigating the immunomodulatory ability of specific microbes. Identification of immunomodulatory microbes and/or metabolic product may have great value in future development of therapeutics, including treatments for autoimmune disorders, inflammatory diseases, and enteric infections.

1.6 *Prevotella* spp. in health and disease

The genus *Prevotella* contains obligate anaerobic Gram-negative bacteria of the Bacteroidetes phylum. Some of the known *Prevotella* species have been reported to be involved in opportunistic infections, while most of them are classically considered to be commensal colonizing different mucosal sites – oral, intestinal, and vaginal[135]. *Prevotella* spp. were found to be a biomarker in one of the three human gut enterotypes[112]. Recent studies have associated the prevalence of *Prevotella* to non-Westerners and agrarian societies who consume a plant-rich diet[136,137] as well as individuals with vegetarian dietary habits[138]. Moreover, it has been shown that *Prevotella* spp. can improve glucose metabolisms stimulated by the intake of prebiotics[139]. Together, these studies suggest *Prevotella* spp. have positive effects on the host and can be considered beneficial microbes.

In contrast, other studies have associated *Prevotella* spp. with autoimmune diseases, insulin resistance and diabetes, and gut inflammation[99,140,141]. Specifically, an overabundance of *Prevotella copri* was noted in new-onset rheumatoid arthritis patients[99]. In mouse models, an altered gut microbiota dominated by a member of the genus *Prevotella* was discovered in NLRP6-deficient mice and was associated with higher susceptibility to chemically-induced colitis[18]. Interestingly, *Prevotella* spp. along with SFB and *Helicobacter* spp. are among the highest IgA-coated bacteria in these mice, which has been interpreted to reflect their immunogenic features[69]. Dysbiotic community derived from *Nlrp6*$^{-/-}$ mice (DysN6) affected

the severity of intestinal inflammation in an immunocompetent host via antigen-specific CD4+ T cells[67]. In addition, this community was characterized by an altered metabolic profile that can suppress inflammasome activation, and consequently reduce IL-18 maturation and antimicrobial peptide secretion[46]. Moreover, studies have shown that intestinal dysbiosis in HIV patient is characterized with increased abundance of *Prevotella* spp., which has been suggested to be the driver of persistent inflammation in the gut[142]. While some studies in IBD patients support the findings in mice[143,144], other studies showed no associations[91]. These data suggest that a *Prevotella*-dominated microbiome may have the propensity to promote inflammation and intestinal dysbiosis, yet the direct functional relevance of increased *Prevotella* colonization is largely unclear.

In summary, there are compelling associations in mice and humans that certain *Prevotella* species can promote inflammatory disease. However, there is a need for more mechanistic and causal studies to demonstrate a potential disease-triggering role for *Prevotella* spp.

1.7 Microbiota-associated immunomodulatory metabolites

The microbiota synthesizes and converts vast numbers of metabolites, however, current limitations in technical approaches limit our knowledge on the number and diversity of microbial metabolites. These include metabolites produced from dietary components, metabolites produced by the host and modified by the gut bacteria, and *de novo* synthesized compounds[145]. It has become clear that the molecules produced through microbiota activity provide important signals to the host and have the ability to shape the immune system. However, observations connecting the microbiota, the metabolome and the immune response have been sporadic and only at the beginning of its discovery[146]. The following section discusses the most studied examples of metabolites shaping the gut-immune axis in the past decade.

1.7.1 Short-chain fatty acids (SCFA)

All animals, including humans, lack the enzymes to digest dietary fibers. These indigestible polysaccharides are fermented in the cecum and in the large intestine by members of intestinal microbiota. The major products of the fermentation process are the so-called short-

chain fatty acids with acetic, propionic, and butyric acids being the most abundant[147]. The colon absorbs SCFAs across the apical membrane in the dissociated form mostly by an anion exchange process with bicarbonate. A small part of undissociated SCFAs may be transported via passive diffusion[148].

As an end product of microbial fermentation of dietary fiber, production of SCFAs in the intestine is firmly dependent on the diet and the microbiota composition. Members of Clostridia and Bacteroidetes have been reported to be main producers of SCFAs. While production of butyrate has been associated to Clostridia, members of the Bacteroidetes have been reported to be a major contributor to acetate production[149,150]. Therefore, the complex and delicate interaction within the microbiota may also control the proportion and levels of SCFAs in the gut lumen.

Over the past few decades, it became evident that SCFAs have an impact on various aspects of the host physiology and play an important role in the maintenance of intestinal health. Apart from being a major energy source for colonocytes, SCFAs play a role in the prevention and treatment of the metabolic syndrome[151,152], certain types of cancer[153], and inflammatory bowel disorders [154–156]. Even though positive anti-inflammatory effects in the treatment of ulcerative colitis and Crohn's disease have been demonstrated over twenty years ago, the molecular mechanism by which SCFAs modulate host immune response to promote homeostasis is still an active, ongoing field of research today.

In the recent years, two major SCFAs signaling mechanisms have been identified; first, the activation of G-protein-coupled receptors (GPCRs) and second, the inhibition of histone deacetylases (HDACs, a class of regulatory proteins that function as inhibitors of gene expression). Several GPCRs, particularly GPR43, GPR41, and GPR109A, have been identified as receptors for SCFAs. Since they sense free fatty acids, GPR43 and GPR41 are also known as free fatty acid receptors (FFARs), FFAR2 and FFAR3, respectively. GPR43 and GPR41 expression have been identified along the entire gastrointestinal tract and in a wide range of immune cells (basophils, neutrophils, monocyte, dendritic cells and mucosal mast cells) suggesting a broad role of SCFAs in immune signaling[157,158]. Probably the most potent anti-inflammatory property of SCFAs is their ability to promote Tregs. A study by Smith and colleagues demonstrated that GPR43 can also be expressed on intestinal Treg cells and that SCFAs stimulate their expansion and IL-10 production[159]. In contrast, another study reported that an effect of SCFAs on cytokine production in T cells is regulated via GPCR-independent mechanism, since the expression of GPR43 in Tregs is not significant[160]. Nevertheless, two independent studies showed that fiber-derived SCFAs

indeed promote the generation of Treg cells, but via inhibition of histone deacetylase (HDAC)[161,162]. Both studies showed a link between SCFAs and Treg cells as mice fed with high-fiber diet or only butyrate displayed an increase in the number of Treg. The increase in Tregs was related to the increased histone H3 acetylation within a genetic locus required for Treg induction, namely Foxp3[161,162]. In addition, SCFAs have been described to promote B cell differentiation into antibody-producing cells[163].

Expressing both receptors, GPR43 and GPR109a, intestinal innate immune cells and IECs are also affected by SCFAs. Acetate and butyrate can act on GPR43 and GPR109a receptors on IECs, respectively, and stimulate them to produce cytoprotective IL-18[63,164,165]. SCFAs-GPR43 signaling was reported to be important for neutrophil chemotaxis and resolution of intestinal inflammation in a model of chemically induced colitis[158,166]. GPR43-deficient mice (*GPR43*$^{-/-}$) showed exacerbated intestinal inflammation in comparison to wild-type (WT) littermates. Feeding WT mice, but not *GPR43*$^{-/-}$mice, with 200 mM acetate in their drinking water resulted in a substantial decrease of intestinal inflammation[158].

Interestingly, SCFA production in the intestine has been shown to have far-reaching consequences for the host, as SCFA have an impact on host physiology even in systemic sites. In rodent models, fermentation of dietary fiber and SCFA production have been causally linked to reduced weight gain and improved glucose tolerance[151,152]. A high-fiber diet (producing high amounts of acetate) suppresses allergic airway disease by enhancing regulatory immune responses[167]. Recent discoveries have suggested significant effects of SCFAs on peripheral as well as the central nervous system. For example, SCFAs impact the numbers and function of the microglia in the brain[168], as well as regulate the permeability of the blood-brain barrier[169]. In addition, SCFAs have been identified as potent regulators of osteoclast metabolism and bone homeostasis[170]. A wide range of effects on host physiology and pathophysiology clearly makes the SCFAs and interesting candidate for development of new treatments for numerous diseases.

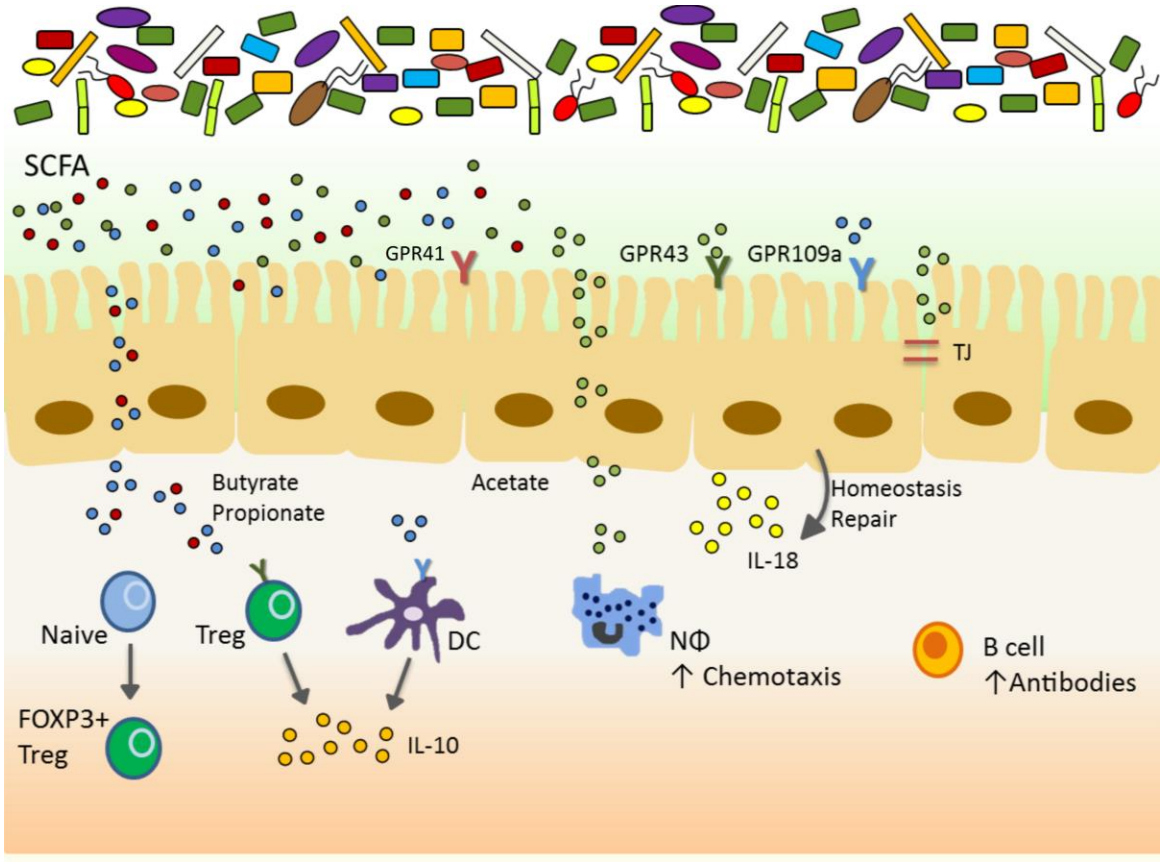


Figure 2: SCFA regulation of intestinal immunity.

SCFA modulate the intestinal mucosal immunity through exerting their effects on various immune and epithelial cells, all expressing distinct G–coupled receptors (GPR). The differentiation of T cells is mediated both by the effects of SCFA on dendritic cells (DC) and the direct act of SCFA on naive T cells, both promoting regulatory immune responses – specifically the expansion of Tregs and IL-10 production. SCFA induce neutrophils migration to an inflammatory site and enhance their phagocytosis. In addition, SCFA regulate intestinal barrier integrity by inducing intestinal epithelial cell secretion of IL-18 and upregulating the expression of the tight junctions (TJ). Effects of SCFA on IgA production in B cells have been as well reported.

1.7.2 Polyamines

Polyamines such as putrescine, spermidine, and spermine are derived from the precursor amino acids arginine and ornithine[171]. They are present in every living cell and play an important role in numerous biological processes, including gene expression, prokaryotic and eukaryotic cell proliferation, response to cellular stress, and metabolism[172]. The colonic lumen contains polyamines found in the diet or exported by enteric bacteria[173]. As germ-free

mice feature increased levels of arginine and diminished levels of polyamines, commensal bacteria have been suggested to play a part in the metabolism of arginine to downstream polyamines[174].

Despite the known role of polyamines in intestinal homeostasis, still not much is known mechanistically about their function. They have been associated with anti-inflammatory functions by acting on various cell types, including macrophages and epithelial cells[175,176]. Furthermore, an increase in polyamine levels in the intestine was shown to enhance the development of intestinal mucosa and recovery from injury[177,178]. They have been shown to exert protective effects against *C. rodentium* driven colitis[179]. In addition, polyamines play a role in the maintenance of intestinal barrier and permeability by altering the synthesis, expression, and stability of tight junction proteins[180,181].

Contrary to these observations, a recent report demonstrated that polyamines inhibit inflammasome signaling and IL-18 production[46]. Interestingly, another metabolite, taurine, was described to counteract the polyamine effects suggesting a fine balance of microbial metabolites play an important role in shaping the immune system[46].

1.9 Experimental models of IBD

CD and UC are complex diseases that originate from an overt immune response to enteric microbiota in the genetically susceptible host[182]. A number of mouse models have been developed to investigate the pathogenesis of IBD and discover targets for treatments. Over the past two decades, they have provided important insights into the immunopathogenesis responsible for the development of intestinal inflammation[183]. Even though no single model captures the complexity of immunopathology of CD and UC, they offer distinct advantages and provide valuable insights into one or more aspects of IBD.

Currently, there are over 50 mouse models of intestinal inflammation[183] and can be grouped into 4 broad categories that include genetically engineered, spontaneous, immune-manipulated, and chemically-induced colitis[184,185]. Some of the most widely and commonly used colitis models include T cells transfer colitis, genetically engineered TNF-α overexpressing (*Tnf*$^{\Delta ARE}$ model)[186] and IL-10 deficient mice models[187], as well as chemically induced dextran sodium sulfate (DSS)[188] and 2,4,6-trinitrobenzene sulfonic acid (TNBS) colitis[189]. Since the DSS colitis model was used throughout the experimental part of this

thesis to study intestinal tissue damage and inflammation, its characteristics and advantages are summarized in the following section.

1.9.1 Dextran sulfate sodium (DSS) colitis

DSS is a sulfated polysaccharide that is directly toxic to the colonic epithelium. Administration of DSS for short periods of time in the drinking water of mice causes injury to the intestine and results in acute inflammation[190]. Inflammation is predominantly limited to the colon and it is characterized by general disruption of the epithelial barrier, including epithelial erosion, loss of crypts, and infiltration of granulocytes[191]. Clinically, diarrhea, bloody stool, weight loss, and eventually death are observed in mice treated with DSS[188,191]. As intestinal inflammation is induced independently of adaptive immune cells (SCID and *Rag2*$^{-/-}$ mice)[192], DSS model has been useful in studying innate immune pathways in colitis induction. As the major feature of DSS colitis is disruption of the intestinal barrier, it is a useful model for studying maintenance of the epithelial integrity and tissue repair after injury[190].
Advantages of this model are simplicity, low cost, reproducibility, rapid onset of inflammation and histopathological similarity to ulcerative colitis[183]. A search in PubMed (June 2018) reveals that over 3000 studies published have used DSS colitis model, making it the most frequently used model.

Despite the success of advancing our understanding of IBD immunopathology, largely attained from numerous studies in animal models, there has been a significant challenge to translate the preclinical research into successful treatments. The rate of translation of efficacious treatment strategies is relatively low, from 60 new therapeutic targets that have been evaluated in over 600 phase I-III clinical studies, only 4 of them have been approved for patient treatment[193]. It has been suggested that the low success rate of generating new treatments for IBD is not only due to limitations of animal models, but mostly due to numerous and diverse genetic predisposing factors and complexity of interactions with the various gut microbiota in IBD. As discussed, IBD constitute very heterogeneous conditions and distinct phenotypes, and as such personalized approach to each patient may allow greater success in treatment[194].

1.10 Aims of the work

The microbiota affects hosts physiology largely by shaping the development of the immune system, the type of immune responses, the metabolism, and by directly preventing the colonization of invading pathogens. Technical advances of culture-independent methods have allowed the extensive characterization of microbial communities in humans and animal models and have established associations between alteration in microbiota composition and a wide range of metabolic disorders, autoimmune and infectious diseases. Regardless of this progress, moving from observational to mechanistic studies and demonstrating cause-effect relationships remains a major challenge in performing these studies as isolation and culture of members of intestinal microbiota is still a critical step.

Recent studies in humans and animal models have associated increased relative abundance of members of Prevotellaceae family within diverse microbial ecosystems with a wide range of inflammatory diseases. Yet, whether these species actively propagate inflammation in these ecosystems remains unknown. Particularly, the role of members of the *Prevotella* genus within the intestinal microbiota and their effects on intestinal homeostasis and inflammation is not completely understood. The detailed investigation of the immunomodulatory properties of *Prevotella* spp. and their potential mechanisms have been restricted by the poor characterization of intestinal *Prevotella* species as well as the lack of diverse intestinal *Prevotella* isolates. As of now, culture collections include three *Prevotella* isolates from the human intestine, while no species isolated from mice are available.

Altogether, these findings bring into a question whether *Prevotella* species are indeed linked to inflammatory disorders or rather a bystander in these microbial communities. Hence, the aim of the present work was to investigate whether presence of *Prevotella* in the intestine promotes inflammatory diseases, in particular intestinal inflammation. Since performance of detailed studies has been hampered by limited availability of intestinal isolates, not only human but from model organisms such as the mouse, the aim of the present work was to isolate distinct species of the *Prevotella* genus from gut microbial communities in mice prone to intestinal inflammation using a step-wise enrichment and targeted isolation. The mouse isolate with the highest genetic similarity to known human intestinal isolates was selected and used as a representative species for investigating the role of *Prevotella* spp. in the intestine. Using wild-type (WT) mice with a defined specific pathogen free (SPF) microbiota composition, and devoid of any *Prevotella* species, we aimed to study the impact of the *P. intestinalis* colonization on the composition and function of the resident microbiota.

Furthermore, the aim of the work was to functionally study the consequence of *P. intestinalis* colonization on the intestinal homeostasis and inflammation using an animal model of chemically induced tissue injury. Investigation of immunomodulatory potential of *P. intestinalis* was performed by global analysis of infiltrating immune cells and cytokine production as well as by using immune-deficient mice models. Members of the intestinal microbiota have been shown to modulate the immune response directly or via production of immuno-modulating metabolites. Whether *Prevotella* immunomodulation capacity comes from its immunogenic properties and/or metabolic activity was evaluated using *in vitro* cell culture assays and targeted metabolome analysis.

Together this comprehensive investigation advanced our understanding of the impact of *Prevotella* spp. on the intestinal ecosystem and the modulation of intestinal homeostasis and inflammation.

Materials

2.1 Experimental mouse models

Wild-type and all transgenic mice, *Rag2*$^{-/-}$ and IL-17A^{GFP} IFN-γ^{Katushka} FoxP3^{RFP} reporter mice used in the study were on the C57BL/6N background. They were all bred and maintained at the animal facilities of the Helmholtz Centre for Infection Research (HZI) under enhanced specific pathogen-free conditions (SPF). All transgenic mice were rederived into SPF microbiota by embryo transfer[37]. *Nlrp6*$^{-/-}$ mice were obtained from Yale University and subsequently bred under conventional housing conditions at the HZI without rederivation. Other used WT mice with different microbiota were purchased either from Janvier Labs (barrier 10C) or were obtained from National Cancer Institute (NCI) and bred under conventional housing conditions at the HZI without rederivation. Germ-free WT mice were bred in isolators (Getinge) in the germ-free facility of the HZI. All experiments were carried out with gender- and age-matched animals.

All mice were provided with sterilized food and water ad libitum. Mice were kept under strict 12 h light cycle and housed in individually ventilated cages (IVC) in groups of up to 5 mice per cage. During the experiment, all mice were kept in airtight ISOcages, containing HEPA-filter, to prevent contamination. All animal experiments have been performed with the permission of the local government of Lower Saxony, Germany.

2.2 Bacterial strains

Table 1 Bacterial cultures used in this study

Bacteria	Source
Prevotella intestinalis	Laboratory stock; Isolated from colonic content of *Nlrp6*$^{-/-}$ mice
Prevotella rodentium	Laboratory stock; Isolated from colonic content of WT mice (Janvier Labs, barrier 10C)
Prevotella muris	Laboratory stock; Isolated from total intestinal content of WT mice (NCI)
Bacteroidetes acidifaciens	DSMZ 100502

2.3 Reagents

2.3.1 Oligonucleotides and probes

Table 2 PCR and qPCR primers used in this study

Oligonucleotide	Sequence
16S_V4Seq	515F:5´AATGATACGGCGACCACCGAGATCTACACT ATGGTAATTGTGTGCCAGCMGCCGCGGTAA
	806R: 5´GGACTACHVGGGTWTCTAAT
16S_SangerSeq	27F: 5´AGAGTTTGATCMTGGCTCAG
	1492R: 5´TACGGYTACCTTGTTACGACTT
16S_qPCR	334F: 5´ACTCCTACGGGAGGCAGCAGT
	514R: 5´ATTACCGCGGCTGCTGGC
P_intestinalis	181F: 5´CGTCCCTTGACGGCATCCGACA
	1032R: 5´CAGCCCCGAAGGGAAGGGGTG
P_rodentium	603F: 5´TGAAATGTCGGGGCTCAACCTTGACAC
	1289R: 5´GCGGCTTTACGGATTGGACGTACG
P_muris	61F: 5´GGCAGCATGACATGTTTTCGGACGT
	642R: 5´CAGTTCGCGCTGCAGGACCG

Table 3 Primer probes used to quantify relative gene expression in this study

Oligonucleotide	Sequence
Il18	Applied Biosystems (Mm00434225_m1)
Casp1	Applied Biosystems (Mm00438023_m1)
Nlrp6	Applied Biosystems (MM00460229_m1)
Ang4	Applied Biosystems (Mm_03647554_g1)
Retnlb	Applied Biosystems (Mm_00445845_m1)
Reg3g	Applied Biosystems (Mm_0044127_m1)
Hprt	F: CTGGTGAAAAGGACCTCTCG; R: TGAAGTACTCATTATAGTCAAGGGCA; Probe:TGTTGGATACAGGCCAGACTTTGTTGGAT

2.3.2 Antibodies

Table 4 Antibodies used in this study

Antibody	Clone	Source
anti-CD45	30-F11	Biolegend
anti-CD3	17A2	Biolegend
anti-CD4	RM4-5, GK1.5	Biolegend
anti-CD8a	53-6.7	Biolegend
anti-CD44	IM7	Biolegend
anti-CD62L	MEL-14	Biolegend
anti-IL-17A	TC11-18H10.1	Biolegend
anti-IFNγ	XMG1.2	Biolegend
anti-MHC class II	M5/114.15.2	Biolegend
anti-CD11b	M1/70	Biolegend
anti-CD11c	N418	Biolegend
anti-Ly6G	IA8	Biolegend
anti-Ly6C	HK1.4	Biolegend
AlexaFluor350 NHS Ester	N/A	Life Technologies

2.3.3 Chemicals, Peptides and Recombinant Proteins

Table 5 Chemicals, peptides, and recombinant proteins used in this study

Reagent	Source
ALBUMIN BOVINE, FRACTION V	MP Biomedicals
Avidin-HRP	BioLegend
Bacto Agar	BD Bioscience
Brain Heart Infusion Broth BHI	Oxoid
Chloroform	Avantor Performance Materials
Collagenase D	Roche
cOmplete Protease Inhibitor Cocktail Tablets	Roche Diagnostics
DEXTRAN SULFATE SODIUM SALT (36000-50000 M.Wt.) Colitis Grade	MP Biomedicals
Dispase	Corning
DNase I	Roche
Dulbecco's Modified Eagle's Medium (DMEM)	Sigma-Aldrich
Dulbecco's Phosphate Buffered Saline (without $CaCl_2$, $MgCl_2$)	Sigma-Aldrich
Dulbecco's Phosphate Buffered Saline (with CaCl2, MgCl2)	Sigma-Aldrich
Ethanol	Avantor Performance Materials
Ethylenediamine tetraacetic acid disodium salt dihydrate (EDTA)	Carl Roth
Fetal Bovine Serum	Sigma-Aldrich
Glycerol	Carl Roth
Glycine	Carl Roth
L-Glutamine	Thermo Fisher Scientific
Hank´s Balanced Salt Solution (HBSS)	Gibco
Horse serum	Sigma-Aldrich
IGEPAL CA-630	Sigma-Aldrich
Ionomycin	Sigma-Aldrich
IsoFlo	Ecuphar
Magnesium chloride $MgCl_2$	Carl Roth
Menadione crystalline	Sigma-Aldrich
Phorbol 12-myristate 13-acetate (PMA)	Sigma-Aldrich
Penicillin-Streptomycin Solution	Thermo Fisher Scientific
Percoll	GE Healthcare
Polymerase Q5 High-Fidelity	NEB
Polymerase TSG	BioBasic

Table 5 continued

Reagent	Source
2-Propanol	Avantor Performance Materials
RNase	AppliChem
Recombinant Mouse IL-18	MBL
RevertAid Reverse Transcriptase	Thermo Fisher Scientific
Roth poly $d(T)_{12-18}$ Primer	Carl Roth
Roti-Phenol/Chloroform/Isoamyl alcohol	Carl Roth
Sheep blood (defibrinated)	Thermo Fisher Scientific
Sodium Acetate solution (3 M, pH 5.2)	Panreac AppliChem
Sodium carbonate Na_2CO_3	Carl Roth
Sodium chloride NaCl	Carl Roth
Sodium Dodecylsulfate solution 20% pure	Panreac AppliChem
Sulfuric acid	Carl Roth
TE buffer (1X) pH 8.0	Panreac AppliChem
Thioglycollate Medium	BD Bioscience
TMB Substrate Set	BioLegend
TRI Reagent	Zymo Research
TRIS	Carl Roth
Triton X 100	Carl Roth
TWEEN 20	Sigma-Aldrich
TWEEN 80	Sigma-Aldrich

2.3.4 Commercial assays

Table 6 Commercial assays used in this study

Commercial Kits	Source
ELISA MAX Standard SET Mouse IL-6	BioLegend
KAPA PROBE FAST qPCR Kit	Kapa Biosystems
KAPA SYBR FAST qPCR Kit	Kapa Biosystems
LEGENDplex Mouse Inflammation Panel	Biolegend
LEGENDplex Mouse Proinflammatory Chemokine Panel	Biolegend
Mix2Seq kit	Eurofins Genomics
Spin Column PCR Product Purification Kit	BioBasic

2.4 Buffers, Mediums and Agars recipes

BHI-S+ (Brain Hearth Infusion Broth + supplements)

Component	Stock	Volume/Mass	Final concentration
BHI	n/a	9.25 g	n/a
Add 225 mL MilliQ water and autoclave for 12 min at 121^{o}C			
FBS	n/a	25 ml	10% (w/v)
Menadione (vit K)	0.5 g/l	250 µl	0.5 mg/l
Filter sterilize into the autoclaved BHI broth, keep away from light, reduce in anaerobic chamber for 2 days before use			

BHI-SVanc+ (BHI-S+ Vancomycin)

Component	Stock	Volume/Mass	Final concentration
BHI	n/a	9.25 g	n/a
Add up to 225 mL with MilliQ water and autoclave for 12 min at 121^{o}C			
FBS	n/a	25 ml	10% (w/v)
Menadione (vit K)	0.5 g/l	250 µl	0.5 mg/l
Vancomycin	7 mg/ml	250 µl	7 µg/ml
Filter sterilize into the autoclaved BHI broth, keep away from light, reduce in anaerobic chamber for 2 days before use			

BHI-SVanc+ agar plates

Component	Stock	Volume/Mass	Final concentration
BHI	n/a	18.5 g	n/a
Add 225 mL with MilliQ water and autoclave for 12 min at 121^{o}C			
Bacto agar	n/a	9 g	n/a
Add 250 mL with MilliQ water and autoclave			
Sheep blood	n/a	25 ml	5 % (w/v)
Menadione (vit K)	0.5 g/l	250 µl	0.5 mg/l
Vancomycin	7 mg/ml	250 µl	7 µg/ml
Add sterile supplements into the autoclaved BHI broth (RT), add together to autoclaved agar (40-50^{o}C), pour 25 ml per petri dish, keep away from light, reduce in anaerobic chamber for 2 days before use			

Buffer A

Component	Stock	Volume/Mass	Final concentration
NaCl	n/a	1.16 g	200 mM
Tris	n/a	2.42 g	200 mM
EDTA- DiNa	n/a	0.74 g	20 mM
Add 100 mL with MilliQ water and set pH to 8			

Hot shot Lysis Buffer

Component	Stock	Volume/Mass	Final concentration
NaOH	10 N	62.5 µl	25 mM
EDTA	0.5 M	10 µl	0.2 mM
Add 25 mL with MilliQ water, pH=12			

Neutralization Buffer

Component	Stock	Volume/Mass	Final concentration
Tris-HCl	1 M	1 ml	40 mM
H_20	n/a	24 ml	n/a
			pH=5

ELISA Coating Buffer

Component	Stock	Volume/Mass	Final concentration
$NaHCO_3$	n/a	8.4 g	100 mM
Na_2CO_3	n/a	3.56 g	34 mM
			Add 1000 mL MilliQ water and set pH to 9.5

ELISA Washing Buffer

Component	Stock	Volume/Mass	Final concentration
PBS	n/a	1000 ml	n/a
Tween 20	n/a	500 µl	0.05% (w/v)

ELISA Blocking Buffer

Component	Stock	Volume/Mass	Final concentration
PBS	n/a	100 ml	n/a
BSA	n/a	1 g	1% (w/v)

Elution Buffer (EB)

Component	Stock	Volume/Mass	Final concentration
Tris-HCl	1 M	0.5 ml	10 mM
H_2O	n/a	45.5 ml	n/a
			pH=8.5

NP-40 Lysis buffer

Component	Stock	Volume/Mass	Final concentration
NaCl	n/a	1.753 g	150 mM
Tris- HCl (pH 8)	500 mM	20 ml	50 mM
IGEPAL	n/a	2 ml	1% (w/v)
			Add up to 200 mL with MilliQ water, before use add 1 tablet of protease inhibitors (Complete) per 50 mL buffer

Fluorescence-activated cell sorting (FACS) Buffer

Component	Stock	Volume/Mass	Final concentration
PBS	n/a	100 ml	n/a
FBS	n/a	1 ml	1% (w/v)

DMEM Complete Medium

Component	Stock	Volume/Mass	Final concentration
DMEM	n/a	445 ml	n/a
FBS	n/a	50 ml	10% (w/v)
L-glutamin	200 mM	5 ml	2 mM
			Filter sterilize, store at 4^{o}C

Bone Marrow-derived Macrophages Medium

Component	Stock	Volume/Mass	Final concentration
DMEM	n/a	315 ml	n/a
FBS	n/a	50 ml	10% (w/v)
L-glutamine	200 mM	5 ml	2 mM
Penicillin (10,000U/ml) - Streptomycin (10,000 µg/ml)	-	5 ml	1% (w/v)
Horse serum	n/a	25 ml	5% (w/v)
L292 supernatant	n/a	100 ml	20% (w/v)
			Filter sterilize, store at 4°C

2.5 Equipment

Table 7 Appliances used in this study

Equipment	Source
Anaerobic chamber	Coy Laboratory
BD LSR	BD Biosciences
BioPhotometer Plus	Eppendorf
Centrifuge 5430 R	Eppendorf
FlexCycler² Thermocycler	Analytik Jena
HERAcell 150i CO2 incubator	Thermo Fisher Scientific
Light Cycler 480 Instrument	Roche Diagnostics
Mainz COLOVIEW System	Karl Storz
Microscope Eclipse TS100	Nikon
Mini-Beadbeater-96	Bio Spec
NanoDrop 1000 Spectrophotometer	Thermo Fisher Scientific
peqTWIST Vortex	VWR
Savant DNA SpeedVac	Thermo Fisher Scientific
Synergy 2 Multi-Mode Reader	Biotek
Thermo Mixer comfort	Eppendorf

2.6 Software and Algorithms

Table 8 Software and Algorithms used for data analysis in this study

Software and Algorithms
FlowJo software
GraphPad Prism v 6.0
R statistical programming environment; R version 3.3.0 (2016-05-03)
Quantitative Insights into Microbial Ecology (QIIME) v 1.8.0
Silva Ref NR database Version 123.1
Greengenes reference database
Ribosomal Database Project (RDP) classifier
OTU picking with UCLUST
FastTree Price
Phyloseq
LEfSe

Methods

3.1 Anaerobic isolation of novel *Prevotella* spp.

Before the start, all materials and reagents were reduced to anaerobic conditions by placing them in anaerobic chamber for a minimum of 48h. The fresh colonic content of conventionally housed donor *Nlrp6*$^{-/-}$ mice was collected in BBL thioglycollate media, weighted and homogenized by vortexing. The fecal content homogenate was further processed in an anaerobic chamber with following gas mixture: 70% nitrogen, 20% carbon dioxide and 10% hydrogen. To remove undigested foods and other bigger particles, the content was filtered through 70 µm sterile filter and diluted to a concentration of 40 mg/ml. Bacteria were isolated by using the most probable number (MPN) technique[195] where homogenized content was diluted in a range in which maximal 30% of wells showed detectable growth. Specifically, 10-fold dilutions (10^{-6} and 10^{-7}) of fecal content homogenate were cultured in a sterile 96-well plate in Brain Hearth Infusion broth (BHI), supplemented with 10% FBS and 0.5 g/l vitamin K (BHI-S+) on 37 ^{o}C for 2 days. To identify *Prevotella*-positive wells, DNA of all 96 wells was isolated via hot shot lysis and screened for *Prevotella* spp. growth by PCR using specie-specific primers. *Prevotella*-positive wells were further subcultured in 5 ml BHI-S+ medium containing vancomycin (BHI-SVanc+) to reduce the contamination of other bacteria and enrich for *Prevotella* spp. *Prevotella*-enriched cultures were further plated on BHI-SVanc-blood agar and single colonies were screened by PCR, using both *Prevotella*-specific primers and sequencing 16S rRNA gene amplicons. Positive colonies were passaged 3 times on agar plates before a pure culture was obtained and glycerol stocks were frozen in -80 ^{o}C.

3.1.1 Hot shot lysis

To identify the isolated bacteria, DNA was isolated via Hot shot lysis. A 4 µl aliquot from each well culture was added to 40 µl of Hot shot lysis buffer and heated for 45-60 min on 95 ^{o}C followed by the addition of 40 µl of Neutralization buffer. The neutralized lysate was further diluted 1:10 in Elution buffer (EB buffer) to reduce the amplification of background DNA present from dead or lysed cells.

3.1.2 *Prevotella* spp. specific polymerase chain reaction (PCR)

Isolated DNA from anaerobic bacterial cultures was tested for *Prevotella* growth using specific PCR primer pair (180F, 1032R) in the following PCR mixture:

Component	Volume (µL)	Final concentration
PCR buffer (10x)	5	1x
$MgSO_4$ (10x)	5	1x
dNTPs	1	200 µM
Forward Primer	1	200 µM
Reverse Primer	1	200 µM
Taq Polymerase	0.2	1U/ 50 µl
Water	35.8	N/A
DNA template	1	5 ng/µl

PCR conditions were as follows:

Cycle Step	Temperature (^{o}C)	Time (s)	# of cycles
Initial denaturation	94	120	1
Denaturation	94	20	30
Annealing	60	20	
Extension	72	60	
Final extension	72	300	1

3.1.3 16S rRNA gene amplification and Sanger sequencing

Bacteria colonies grown on agar plates were identified by Sanger sequencing. Multiple colonies were picked with sterile inoculation loops directly in Hot shot lysis buffer. Isolated DNA served as a template for 16S rRNA amplification in a PCR reaction using specific primers (16S_27F, 16S_1492R) and following PCR conditions:

Step	Temperature (°C)	Time (s)	# of cycles
Initial denaturation	94	120	1
Denaturation	94	20	11
Annealing	61	20	
Extension	72	60	
Denaturation	94	20	26
Annealing	55	20	
Extension	72	60	
Final extension	72	300	1

16S rRNA amplicons were purified using Spin Column PCR Product Purification Kit according to the manufacturer's instructions and subsequently sequenced by Eurofins Genomics using Mix2Seq kit.

3.2 Microbiota manipulation

3.2.1 *Prevotella intestinalis* mice colonization

For every experiment, fresh *P. intestinalis* culture was grown anaerobically (70% N_2, 20% CO_2 and 10% H_2) from a frozen glycerol stock in BHI-S+ medium on 37 °C for 2-3 days. All mice (unless indicated differently) were colonized at age of 4-5 weeks with 200 µl of freshly grown *P. intestinalis* culture (OD_{600}=0,25) via oral gavage.

3.2.2 *Prevotella* spp. competition

Three *Prevotella* species, *Prevotella intestinalis*, *Prevotella rodentium,* and *Prevotella muris*, were grown anaerobically in BHI-S+ medium on 37 ^{o}C. All three cultures were grown to an OD_{600}=0.25, mixed in 1:1:1 ration and co-transferred together by oral gavage in mice recipients. Before and after colonization feces samples were collected on different days and *Prevotella* spp. colonization kinetics was examined by quantitative PCR and 16 rRNA sequencing.

3.3 Fecal bacteria DNA isolation

Fresh stool samples or intestinal content were collected from mice and immediately stored at -20 ^{o}C. For DNA based 16S rRNA gene sequencing, DNA was extracted according to established protocols using a method combining mechanical disruption (bead-beating) and phenol/chloroform based purification (Turnbaugh et al., 2009). Briefly, samples were suspended in a solution containing 500 µl of DNA extraction buffer, 200 µl of 20% SDS, 500 µl of phenol:chloroform:isoamyl alcohol (24:24:1) and 100 µl of 0.1 mm diameter zirconia/silica beads. Samples were then homogenized twice mechanically using Mini-Beadbeater-96 for 2 min. After centrifugation (8,000 rpm for 5 min at 4 °C) of homogenized feces/fecal content, the aqueous phase was transferred to a new 1.5 ml tube and extraction was repeated by adding 600 µl of phenol/chloroform/isoamyl alcohol and centrifuging at 12,700 rpm for 5 min at 4 °C. Upper aqueous phase containing DNA was transferred into a fresh tube and further precipitated in 600 µl ice-cold 2-propanol with 60 µl of 3M sodium acetate solution at -20 °C for a minimum of 1h. To yield a DNA pellet, samples were centrifuged at 12,700 rpm for 20 min at 4 °C and subsequently washed in 1 ml of 70% ethanol. Finally, the crude DNA pellets were dried in a vacuum centrifuge for 10 min and resuspended in 200 µl of 1xTE buffer. Dissolved DNA samples were treated with 100 mg/ml RNase and column purified to remove PCR inhibitors. The isolated fecal microbial DNA was stored at -20 °C until further analysis.

3.3.1 DNA isolation from mucosa-associated bacteria

Collected colons were opened longitudinally and content was removed by washing two times in sterile 1xPBS, or until all visible content was removed. Mucus associated bacteria were detached from the intestinal wall in three 1-min washes in 12.5 ml 0.1% (w/w) Tween 80 in 1xPBS by vigorously shaking the tube. The washes were pooled and then centrifuged at 4,700 rpm, for 20 min at 4 °C to pellet the cells. The bacterial pellet was further resuspended in DNA extraction buffer (500 µl Buffer A and 200 µl of 20% SDS) and DNA was isolated using standard fecal bacterial DNA isolation protocol.

3.4 16S rRNA microbial community sequencing

Sequencing of fecal DNA was performed in the Genome Analytics Platform at Helmholtz Center for Infection Research.

Amplification of the V4 region (F515/R806) of the 16S rRNA gene was performed as previously described protocols[196]. Samples were sequenced on an Illumina MiSeq platform (PE250). Barcode-based demultiplexing was performed using IDEMP software with default parameters (https://github.com/yhwu/idemp). Obtained reads were assembled, quality controlled and clustered using Usearch8.1 software package (http://www.drive5.com/usearch/). Briefly, reads were merged using -fastq_mergepairs –with fastq_maxdiffs 30 and quality filtering was done with fastq_filter (-fastq_maxee 1), minimum read length 200 bp. The OTU clusters and representative sequences were determined using the UPARSE algorithm[197], followed by taxonomy assignment using the Silva database v128[198] and the RDP Classifier[199] with a bootstrap confidence cutoff of 80% performed by using QIIME v1.8.0[200]. OTU absolute abundance table and mapping file were used for statistical analyses and data visualization in the R statistical programming environment package phyloseq[201]. To determine bacterial OTUs that explained differences between microbiota settings, the LEfSe method was used[202]. OTUs with Kruskal-Wallis test < 0.05 and LDA scores > 4.0 were considered informative.

3.5 Induction of acute DSS colitis

Acute colitis was induced by adding Dextran Sodium Sulfate (DSS) in sterilized drinking water of 10-11 weeks old WT and gene-deficient mice. Mice were given 2.1% (w/v) DSS in drinking water for 7 days, followed by 5 days of access to regular drinking water. During the course of DSS treatment fresh DSS solution was prepared and replaced on day 0 and day 4. Mice were monitored daily by measurement of body weight and clinical assessment, including stool consistency and detection of blood in the stool. Animals which lost 20% or more of their initial body weight were euthanized by carbon dioxide asphyxiation and/or cervical dislocation.

3.6 Sacrifice and dissection of mice

All procedures were performed according to the animal protection act and animal suffering was kept to an absolute minimum. Experimental samples were collected either in steady state (day 0) or during inflammation (day 7 of DSS treatment). Mice were euthanized by CO_2 inhalation, and death of the animals was confirmed by controlling the corneal and interdigital reflexes. For different analysis, colon, colon fecal content, cecum, cecum fecal content, small intestine, small intestine fecal content, and/or mesenteric lymph nodes were collected.

3.6.1 Colon tissue sampling

Colons were removed by making cuts at the proximal part connecting to the cecum and at the rectal part. Excised colons were divided into distal and proximal colon. Three centimeters of each part was cut open longitudinally, gently washed in 1xPBS to remove the fecal content, and further divided longitudinally into two samples for further processing - tissue homogenates (protein) preparation and RNA isolation.

3.7 Tissue homogenates preparation

One half of proximal and distal colon was collected in 1 ml NP-40 lysis buffer with protease inhibitors and 1.0 mm diameter zirconium/glass beads and always kept on ice. Tissue weight was recorded and samples were further mechanically homogenized using Mini-Beadbeater-96 (Biospec). Protein extracts were centrifuged at 10,000 rpm for 5 min at 4 °C and the supernatants were stored at -80 °C until further analysis.

3.8 Enzyme-linked immunosorbent assay (ELISA)

Enzyme-linked immunosorbent assays (ELISA) were performed to determine concentration of different pro-inflammatory cytokines (IL-6 and IL-18) in colonic tissue and in supernatants of stimulated bone marrow derived macrophages (BMDM). The protocols for all measured cytokines were identical and were carried out according to the manufacturer´s instructions.
One day prior to the assay flat-bottom 96-well plates were coated with 25 µL of capture antibody diluted in 1x Coating buffer and incubated overnight at 4 °C. The following day plates were washed 3 times with 120 µL Washing buffer and blocked for 1 h on RT with 50 µL of Assay diluent to avoid unspecific binding Diluent. After washing, 25 µL of each sample and standard dilutions were prepared as stated by the manufacturer in the respective sample buffer or cell medium, which also served as a negative control. Twenty five µL of each sample (diluted if needed) and standard 2-fold dilutions were added to respective wells and incubated for 2 h on RT. All standards, negative controls and samples were done in duplicates. After the incubation, the plates were washed 3 times with 120 µL Washing buffer and 25 µL detection antibody diluted in Assay diluent was added to each well. After 1h incubation at RT, the plates were washed 3 times and 25 µL of avidin-conjugated horse radish peroxidase antibody (HRP) was added to each well. After 30 min incubation at RT the plates were washed 5 times with 120 µL of Washing Buffer. Subsequently TMB substrate solution was prepared by mixing the provided solutions A and B in a 1:1 ratio, and 25 µL was added to each well. Samples were kept in the dark until the desired color has developed. Reaction was stopped by adding 25 µL of 2N H_2SO_4. The absorbance was measured using ELISA plate reader - Synergy 2 analyzer on two wavelengths - 450 nm and

570 nm. The standard values were used to generate a linear standard curve based on which the sample concentrations were calculated.

3.9 LEGENDplex immunoassay

LEGENDplex immunoassay was performed to measure distinct cytokines and chemokines, either in intestinal tissue homogenates or cell culture supernatants. According to the manufacturer´s instructions, samples were incubated in polypropylene microfuge tubes with different size beads set conjugated with distinct capture antibodies, together with assay buffer and a biotinylated detection antibody cocktail. Sample tubes were covered with aluminum foil to protect from light, and were shaken for 2 h on RT. After washing, Streptavidin-phycoerythrin (SA-PE) was added and incubated on RT for additional 30 min while shaking. Finally, the samples were washed, centrifuged at 1,800 rpm for 10 min, and resuspended in FACS buffer. The samples were then acquired on flow cytometer and different cytokine were differentiated by size and internal fluorescence intensity. The concentration of a particular cytokine was determined using a standard curve generated in the same assay.

3.10 Total RNA isolation

One half of proximal and distal colon was sampled and collected in 1 ml TRI reagent containing 1.0 mm diameter zirconium/glass beads and always kept on ice. After mechanical homogenization using Mini-Beadbeater-96, samples were incubated for 5 min at RT to permit the complete dissociation of nucleoprotein complexes. Next, samples were centrifuged at 300 g for 3min at 4 °C and the supernatant was transferred to a new Eppendorf tube prefilled with 200 µL of chloroform. The mixture was shaken vigorously by vortexing for 15 s and incubated at RT for 3 min. Samples were further centrifuged at 12,000 g for 15 min for phase separation. The upper aqueous phase containing RNA was subsequently transferred into a fresh Eppendorf tube and 500 µL of 2-propanol was added for RNA precipitation. The mixture was vortexed and incubated overnight at -20 °C. The next day samples were centrifuged at 12,000 g for 30 min at 4 °C to yield a RNA pellet, followed

by two washes with 1 mL of 75% ethanol and centrifugation at 7,500 g for 5 min at 4 °C. Finally, ethanol was discarded and the air-dried pellets were resuspended in 80 µL of RNase-free water. Total RNA concentration was determined by spectrophotometry using Nanodrop 1000. Samples were stored at -80 °C until further analysis.

3.11 RNA-Seq analysis

The RNA-Seq data processing and analysis was performed with the help of Eric J.C. Galvez, a bioinformatician within Microbial Immune Regulation research group. Total RNA isolation from distal colonic tissue was performed as described in 3.10 section. RNA integrity was measured in a Bioanalyzer (Agilent Technologies, USA) and samples were selected according to RNA Integrity Number (RIN) > 8.5. Isolation of mRNA was performed with Dynabeads mRNA DIRECT Micro Kit (Ambion, USA) using 1ug of total RNA. Furthermore, cDNA synthesis, fragmentation and sequencing library preparation were done using ScriptSeq v2 RNA-Seq Kit (PCR 15 cycles) (Illumina, USA). Sequencing was performed through Illumina Hi-Seq 2000 platform in single end mode for 50 bp. An average of 18 Million of reads per sample (n=8 was obtained). Reads were quality filtered using Trimmomatic with the following parameters: (LEADING:3, TRAILING:3, SLIDINGWINDOW:4:15, MINLEN:35, HEADCROP:3). After quality control, the reads were aligned to the mouse reference genome (mm10) using STAR. Reads count to each gene was evaluated using HTseq. Normalization and differential expression were quantified using the DEseq2 package. Differential expressed gene networks were analyzed with Consensus Path DB-mouse webserver. Data were visualized using ggplot2 R library.

3.12 Complementary DNA (cDNA) synthesis

One microgram of total RNA was used to generate cDNA using RevertAid Reverse Transcriptase, and polyd$(T)_{12\text{-}18}$ primer. Reverse transcription was done as follows:

Step	Component	Volume/Mass	Final concentration
Step 1	RNA	1 µg	1 µg
	Oligo(dT)$_{12-18}$	1 µL	100 pmol
	Add up to 10 µL water and incubate for 5 min at 65 °C		
Step 2	5X Reaction Buffer	4 µL	1x
	dNTP Mix	2 µL	1 mM
	RevertAid Reverse Transcriptase	1 µL	200 U
	Water	3 µL	N/A
	Add 10 µL of reaction mix to RNA and incubate for 60 min at 42 °C		
Step 3	Incubate for 5 min at 85 °C		

Samples were afterwards cooled down (4 °C), diluted 1:10 in water and stored at -20 °C until further analysis.

3.13 Quantitative PCR (qPCR)

Gene expression analysis was performed using quantitative PCR method. Gene-specific probe sets for Casp1, Il18, Ang4, Retnlb, Reg3g, and Hprt were used. Using Kapa Probe Fast qPCR Kit, following 10 µL reactions were performed:

Component	Volume (µL)	Final concentration
PCR-grade water	0.25	N/A
KAPA PROBE FAST qPCR Master Mix (2x)	5	1x
Primer/probe (20x)	0.5	1x
Template cDNA	4.5	N/A

All samples were measured in duplicates on a LightCycler 480 instrument. qPCR conditions were as follows:

Cycle Step	Temperature (°C)	Time (s)	# of cycles	Analysis mode
Preincubation	95	60	1	none
Amplification	95	3	40	Quantification
	60	30		
Cooling	37	60	1	none

Results were exported and gene expression was analyzed using the ΔCt method with Hprt serving as the reference housekeeping gene.

3.14 Isolation of lamina propria lymphocytes (LPLs) and flow cytometry

To isolate lamina propria lymphocytes, density gradient centrifugation using Percoll was done as previously described[203]. Intestinal tissues and mesenteric lymph node (MLN) were collected on day 0 and day 7 of DSS colitis. Colon and small intestine (SI) were open longitudinally and fecal content was removed by washing in 1xPBS. All visible Payers patches in the SI were carefully removed. To remove the epithelial layer, both colon and SI, were shaken in 1xHBSS containing 2 mM EDTA for 20 min at 37 °C. Tissues were then cut into small pieces and incubated with 5 ml digestion solution (DMEM containing 1% fetal bovine serum (FBS), 0.25 mg/ml collagenase D, 0.5 U/ml dispase and 5 mg/ml DNase I) in a shaker for 20 min at 37 °C. Digested tissues were filtered through 70 µM cell strainer and DMEM + 5% FBS was added to inactivate the enzymes. The digestion step was repeated. After centrifugation, cells were resuspended in 4 mL of 40% Percoll and carefully overlaid on 4 mL of 80% Percoll. Percoll gradient separation was performed by centrifugation at 450 g for 25 min at 25 °C. Cells in the interphase, LPLs, were collected, counted and suspended in staining buffer. The following antibodies were used: anti-CD45 (30-F11), anti-CD3 (17A2), anti-CD4 (RM4-5, GK1.5), anti-CD8a (53-6.7), anti-CD44 (IM7), anti-CD62L (MEL-14), anti-MHC class II (M5/114.15.2), anti-CD11b (M1/70), anti-CD11c (N418), anti-Ly6G (IA8), anti-

Ly6C (HK1.4). Live from dead cells were distinguished using AlexaFluor-350 NHS Ester staining. Flow cytometry analysis was performed using a BD LSR Fortessa and data were analyzed with FlowJo software.

For detection of intracellular cytokines and transcription factors, isolated LPL cells were cultured in enriched DMEM media containing 10% FBS, antibiotic cocktail (100U/ml penicillin, 100 µg/ml streptomycin) and 2 mM/ml L-Glutamine for 3h at 37^{o}C. *Ex vivo* stimulations were carried out in the presence of 50 ng/ml phorbol 12-myristate 13-acetate (PMA) and 1µg/ml Ionomycin. For intracellular cytokine staining, cells were fixed and stained using the Foxp3-staining kit (eBioscience) according to the manufacturer's instructions. Antibodies used for staining were anti-IFNg (XMG1.2) and anti-IL-17A (TC11-18H10.1).

3.15 Colonoscopy

Colonoscopy was performed on anesthetized mice using a high-resolution mouse video endoscopic system ('Coloview', Carl Storz, Tuttlingen, Germany). The severity of colitis was scored in a blinded manner using Murine Endoscopic Index of Colitis Severity (MEICS), which is based on five parameters: granularity of mucosal surface (0-3); vascular pattern (0-3); translucency of the colon mucosa (0-3); visible fibrin (0-3); and stool consistency (0-3) (Becker et al., 2007). Murine endoscopic colitis scores are described in Table 9.

Table 9 Murine endoscopic colitis scoring

Score	Granularity of mucosal surface	Vascular pattern	Translucency of the colon mucosa	Visible fibrin	Stool consistency
0	none	normal	transparent	none	normal, solid
1	moderate	moderate	moderate	little	still shaped
2	marked	marked	marked	marked	unshaped
3	extreme	bleeding	intransparent	extreme	spread

3.16 Histological Evaluation

Preparation of intestinal tissue samples for histopathological examination was carried out by Mouse Pathology core facility at Helmholtz Center for Infection Research. Briefly, intestinal tissue samples were placed (cecum) or rolled up to “swiss roles” (colon and small intestine) in histology cassettes, fixed in 4% neutrally buffered formaldehyde for a maximum of 48 h, and stored in 70% ethanol until processing. Samples were further embedded in paraffin according to standard histological procedures and sections of 3 µm thickness were stained with hematoxylin-eosin (HE). Stained sections were evaluated under a light microscopy in a blind manner.

3.16.1 DSS scoring

The histological scoring used to evaluate the severity of colitis was adapted from the TJL-score developed by The Jackson Laboratory[204]. The alteration of the scores has been previously described[205]. The colon was divided into a proximal, middle and distal section, each of about the same size. The three sections were scored for the general criteria: severity, degree of ulceration, degree of edema, degree of goblet cell dysplasia, and percentage of area involved as described in Table 10. The scores were added up to a total of up to 15 per section, or up to 45 per complete colon sample (sum of the scores of the three sections).

Table 10 Histological scoring of colitis severity

Score	Severity	Ulceration	Edema	Goblet cell dysplasia	Area involved
0	no alterations	no ulcer	no edema	no dysplasia	0%
1	mild alterations, focally small or widely separated multifocal areas of inflammation limited to the lamina propria	1-2 ulcers (involving up to a total of 20 crypts)	mild epithelial or submucosal edema (less than the muscular layer in thickness)	few goblet cells with slightly increased size	30%
2	moderate alterations, multifocal or locally extensive areas of inflammation extending to the submucosa	1-4 ulcers (involving a total of 20-40 crypts)	mild epithelial edema associated with mild submucosal edema or more moderate submucosal edema (1 to 2 times as thick as the muscular layer)	various, irregular sizes of goblet cells	40-70%
3	severe alterations, inflammation extended to all layers of the intestinal wall or entire intestinal epithelium destroyed	any ulcers that exceed the previous	every edema more extensive than the previous	ballooned goblet cells, crypt dilation and goblet cell depletion	>70%

3.17 Treatment of mice with recombinant mouse IL-18 (rIL-18)

Each mouse received intraperitoneal (i.p.) injections of 200 ng of mouse recombinant IL-18 suspended in 200 µL sterile PBS for 8 consecutive days, starting 1 day before

administration of DSS in drinking water. Control mice were administered daily with 200 µL of sterile PBS.

3.18 Metabolite measurements

3.18.1 SCFAs extraction and measurements

SCFAs were measured and analyzed by Dr. Meina Neumann-Schaal from Leibniz institute DSMZ-German Collection of Microorganisms and Cell Cultures.
Serum and fecal content samples were snap-frozen in liquid nitrogen and stored at -80 °C until further processing. For SCFAs extraction colonic and cecal content (50-100 mg) was resuspended in 600 µl water spiked with internal standard (2 µl o-cresol/250 ml) and 60 µl 65% HPLC-grade sulfuric acid per 50 mg fresh weight and mixed vigorously for 5 min. 400 µl of the mixture were extracted with 200 µl of tert-butyl methyl ether., and the ether phase was analyzed by GC-MS as described previously[206]. Standard curves of organic acids were used for external calibration.

3.18.2 Metabolome extraction

Metabolites were extracted using an adapted protocol[207]. Briefly, cecal content (50-100 mg) was collected in tubes containing glass and ceramic beads mix (Bertin-corp) and appropriate volume (1000 µL/100 mg cecal content) of -20 °C ice-cold extraction fluid 1 (methanol+H_2O, 4+1) containing 32 µM $U^{13}C$ ribitol as internal standard was added. Homogenization was performed with a Retsch mill (3x 2 min at 30 Hz/sec). An appropriate volume (500 µL/100 mg cecal content) of extraction fluid 2 (H_2O containing 2 µg/mL D^6-glutaric acid) was added and samples were mixed for 10 sec. Chloroform (800 µL/100 mg cecal content) was added and samples were again mixed for 30 sec prior agitation for 15 min at 1400 rpm and 4 °C in a tube shaker. After centrifugation for 5 min at 4 °C and 13.000 rpm, 60 µL of the upper polar phase was transferred to a GC vial with micro insert. Solvents were evaporated at 4 °C in a rotary vacuum evaporator until total dryness. Sample tubes were capped and stored at -80 °C until further processing.

3.18.3 Targeted metabolome GC-MS analysis

Targeted metabolome measurements and analysis was performed with by Dr. Kerstin Schmidt-Hohagen from the Department of Bioinformatics and Biochemistry at Technische Universität Braunschweig

Online metabolite derivatization was performed using an Axel Semrau Autosampler. Dried polar metabolites were dissolved in 15 µL of 2% methoxyamine hydrochloride in pyridine at 40°C under shaking. After 90 min, an equal volume of N-methyl-N-(trimethylsilyl)-trifluoracetamide (MSTFA) was added and held for 30 min at 40 °C.

Sample (1 µL) was injected into an SSL injector at 270 °C in splitless mode. GC-MS analysis was performed using an Agilent 7890A GC equipped with a J&W 30m DB-35MS capillary column (0.25 mm inner diameter, 0.25 µm film thickness). Helium was used as carrier gas at a flow rate of 1.0 mL/min. The GC oven temperature was held at 90 °C for 1 min and increased to 320 °C at a rate of 15°C/min and held at that temperature for 8 min, resulting in a total run time of 25 min per sample. The GC was connected to an Agilent 5975C MS operating under electron impact ionization at 70 eV. The transfer line temperature was set to 280 °C. The MS source was held at 230 °C and the quadrupole at 150 °C. The detector was operated in SIM mode. The detailed settings for each metabolite are summarized in Table 11. Tuning and maintenance of the GC-MS was done according to the supplier´s instructions, an automated tuning routine was applied every 150 injections. Data processing was done using the MetaboliteDetector software[208].

Table 11: Settings applied in SIM mode.

Metabolite	Derivatization	Ions
Cadaverine	4TMS	130.0, 174.0, 375.0
Histamine	3TMS	174.0, 226.0, 312.0
Putrescine	4TMS	174.0, 214.0, 361.0
Spermidine	5TMS	116.0, 144.0, 156.0
Spermine	6TMS	144.0, 174.0, 329.0
U13C-Ribitol (IS)	5TMS	207.0, 220.0, 323.0

3.19 Bone marrow-derived macrophage (BMDM) preparation

Bone marrow cell suspensions were isolated by flushing femurs and tibias of 8- to 12-week-old WT or TLR4-deficient mice with ice-cold, sterile 1x PBS. Cells were further centrifuged at 1500 rpm for 10 min at 4 °C, resuspended in BMDM Medium and counted. Ten million cells were seeded per 10 cm petri dish in 10 ml BMDM Medium and cultured in a humidified incubator at 37 °C, 5% CO_2. Every 2 days medium was replaced with fresh, pre-warmed BMDM Medium. After 6 days, adherent cells were washed in ice-cold, sterile 1x PBS and incubated at 4 °C for 10 min in 3 ml of 50 mM EDTA/PBS to detach them from the surface. Next, cells were harvested by gently scraping and washed in additional 25 ml PBS. Fifty thousand cells were seeded in each 96-well.

3.19.1 Preparation of L292 cell supernatants

L292 cells were grown in 20 ml of DMEM Complete (DMEM + 10% FBS + L-glutamine) per T175 flasks, in a humidified incubator at 37 °C, 5% CO_2. After washing with sterile 1x PBS, fully confluent cells culture was detached from the surface using 2-3 ml of 1xTrypsin/EDTA and centrifuged at 1,500 rpm, for 10 min, at 4 °C. Cells were split in 1:4 ratios into new T175 flasks, and at fully confluency, the supernatant was collected every 2 days and stored at -20 °C until further use for BMDM Medium preparation.

3.20 Preparation of bacterial extracts

Fecal content (colon and cecum) from SPF and SPF+*P. intestinalis* mice were suspended in sterile PBS and filtered through 70 μm cell strainer (BD Falcon). Homogenized content was normalized to 0.5 mg/ml and centrifuged for 5 min at 10,000 rpm. Supernatant were collected for BMDM stimulation (Secreted microbial component) and bacterial pellet was suspended in 1 ml PBS with $CaCl_2$ and $MgCl_2$, freeze/thawed 3 times, sonicated for 2 minutes and incubated at 65 °C for 1 hour. Cell lysate was centrifuged and pellet was suspended in 1 ml DMEM and 10 μl was used for BMDM stimulation (Microbial community)

for 12 h. *P. intestinalis* and *B. acidifaciens* cultures at OD_{600}=0.2 were centrifuged and bacterial pellets were lysed as described for microbial community.

3.21 Statistical analysis

Statistical analysis was performed using GraphPad Prism 6 program (GraphPad Software, Inc.) and R v3.3.0. Data are expressed as mean ± SEM (Standard error of mean). Differences were analyzed by Student's t test and ANOVA. P values indicated represent an unpaired nonparametric Mann-Whitney or two-way ANOVA by Tukey´s multiple comparison analysis. The permutational multivariate ANOVA analysis of variance (ADONIS) was computed with 999 permutations. In addition to p value, for ADONIS tests an $R^2 > 0.1$ (effect size, 10%) was considered as significant. P values ≤ 0.05 were considered as significant: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Results

4.1 Isolation and characterization of distinct *Prevotella* spp. from mouse intestine

Alteration in the microbiota of some lines of *Nlrp6*$^{-/-}$ mice renders them more susceptible to chemically-induced intestinal inflammation and a high relative abundance of unknown members of the family Prevotellaceae was identified by 16S rRNA gene sequencing in mice with high disease susceptibility[18]. In addition, we identified that different species of the genus *Prevotella* were also highly abundant in other colitogenic communities of WT mice from distinct commercial vendors[67]. Yet, whether these species actively propagate inflammation in the intestine remained to be investigated. In order to experimentally address these questions, we attempted to isolate novel *Prevotella* species from the colon content of these mouse lines using a step-wise enrichment and targeted isolation procedure under strictly anaerobic conditions (Figure 3).

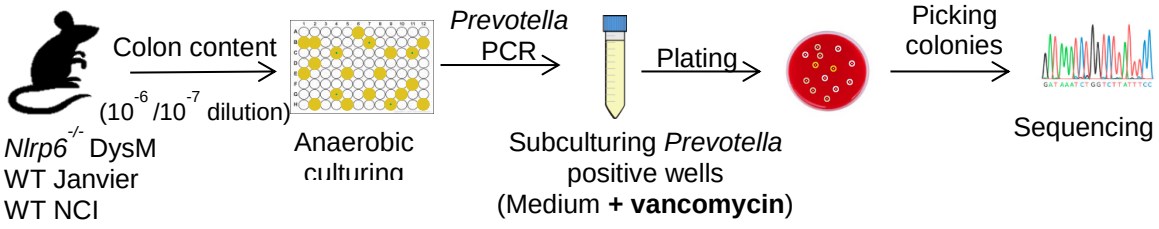


Figure 3: Isolation of *Prevotella* spp.

Scheme of the step-wise enrichment and targeted isolation procedure of *Prevotella* spp. under anaerobic conditions.

The cultivation effort yielded three new species belonging to the genus *Prevotella* based on the comparison of their 16S rRNA genes to other described *Prevotella* species (Figure 4). Based on genotypic characterization we propose that the isolates belong to novel bacterial taxons within the genus *Prevotella*, for which the names *Prevotella intestinalis, Prevotella rodentium, and Prevotella muris* are proposed. Out of these three species, one *P. intestinalis* (OTU_16) showed the highest similarity to the most prevalent human intestinal *Prevotella* species, namely *P. copri* [209].

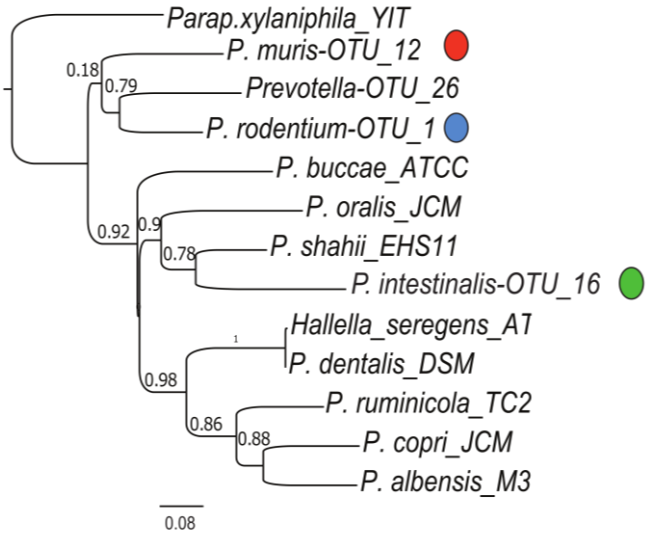


Figure 4: Phylogenetic characterization of novel *Prevotella* isolates

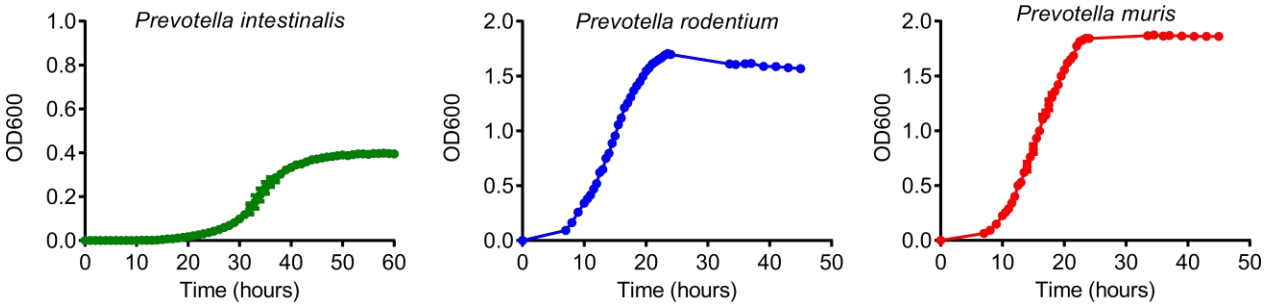
Phylogenetic tree based on 16S rRNA sequences showing the positions of novel *Prevotella* species within the genus *Prevotella*.

Isolated species demonstrated distinct *in vitro* growth rates as well as distinguishable colonies forms and sizes on agar plates. While *Prevotella rodentium and Prevotella muris* demonstrated optimal growth in BHI-S medium (highest OD_{600} *P. rodentium* =1.703, *P. muris*=1.878), *P. intestinalis* cultures are characterized by a low turbidity with highest OD_{600} ≤0.414) suggesting distinct *in vitro* growth requirements (Figure 5A). *P. intestinalis* demonstrated the slowest growth rate, reaching the stationary phase after 58 h, whereas stationary phase was reached by *P. rodentium* and *P. muris* at 24 h and 34 h of growth, respectively.

After 72 h of growth at 37 °C on BHI+blood+vitamin K-agar plates under anaerobic conditions, *P. intestinalis* colonies are circular, pinpoint and translucent to slightly opaque,

whereas *P. rodentium* forms circular, small to moderate, convex, shiny grey colonies (Figure 5B). *P. muris* growth was not detectable in BHI+blood+vitamin K-agar plate, however it forms circular, small to moderate, shiny, creamy colonies on BHI+FBS+vitamin K-agar plates (Figure 5B).

A



B

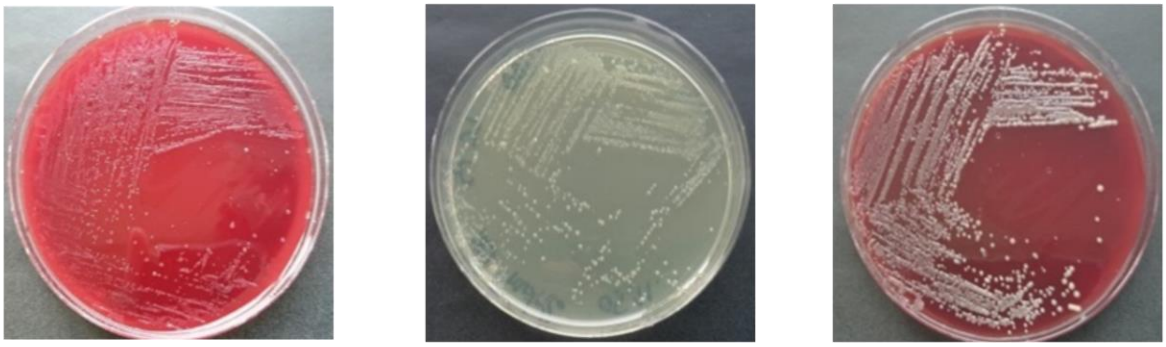


Figure 5: Distinct growth rate and phenotypical differences of novel *Prevotella* isolates

Prevotella growth curves in BHI-S medium (A) and colony growth on BHI+blood+vitamin K- or BHI+FBS+vitamin K-agar plates (B).

A detailed comparative genome analysis of new *Prevotella* species was performed by Eric J.C. Galvez as a part of his PhD research project (data unpublished). In brief, whole genome sequencing and assembly functionally assigned 72% of the genomes (in average) to known features. Interestingly, *P. intestinalis* presented the highest number of unique features, some of them being potential virulence factors such as metalloproteases (i.e. peptidase M6) and cysteine-type peptidase.

4.2 Impact of *Prevotella* spp. colonization on intestinal ecosystem

4.2.1 Colonization of WT SPF and *in vivo* fitness of *Prevotella* spp.

To study the impact of *Prevotella* spp. on the host, we colonized specific pathogen free (SPF) WT mice, devoid of any *Prevotella* species, by single oral gavage of freshly grown culture. After 4-5 weeks *Prevotella* colonization was determined by analyzing fecal microbiota composition using 16S rRNA gene sequencing. Interestingly, all *Prevotella* spp. colonized SPF mice in high relative abundances; *P. intestinalis* (43.4% +/- 5.8, mean +/- SEM), *P. rodentium* (30.4% +/- 2.9, mean +/- SEM), and *P. muris* (24.4% +/- 2.5, mean +/- SEM) (Figure 6A). In contrary to low *in vitro* fitness, *P. intestinalis* colonized SPF mice in the highest relative abundance in comparison to other *Prevotella* species.

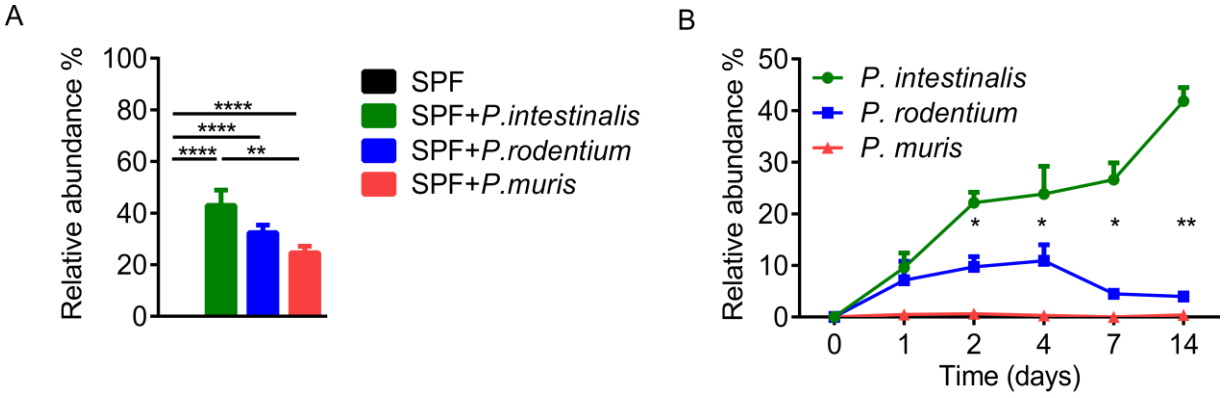


Figure 6: *Prevotella* spp. *in vivo* growth and interspecies competition in WT SPF mice
Relative abundance of *Prevotella* species in feces of WT SPF mice 4 weeks after colonization, as quantified using 16S rRNA gene sequencing (A). SPF mice were colonized with equal amounts of all three *Prevotella* isolates and interspecies competition was determined using 16S rRNA gene sequencing *(P. intestinalis* vs *P. rodentium* statistical test) (B). Data shown as mean ± SEM. P values indicated) represent an unpaired nonparametric Mann-Whitney *p < 0.05; **p < 0.01; ***p< 0.001; ****p< 0.0001.

In order to compare the fitness of the *Prevotella* isolates between each other *in vivo* and to understand whether they occupy the same niche in the intestine, we performed an

interspecies *in vivo* competition assay by colonizing SPF mice with the same amounts of all three *Prevotella* species. Analysis of relative abundance of *P. intestinalis, P. rodentium,* and *P. muris* on day 0, 1, 2, 4, 7, and 14 days post colonization clearly demonstrated the interspecies competition *in vivo*. Relative abundance of *P. intestinalis* was the highest throughout the time course of the experiment, outcompeting the other two *Prevotella* species (Figure 6B). While *P. muris* abundance was under the detection limit, *P. rodentium* demonstrated the ability to coexist with dominant *P. intestinalis* species, however in significantly lower abundance than when colonizing SPF microbiota alone (Figure 6A and 6B. These data suggest *Prevotella* species share similar metabolic niches within the mouse intestine, with *P. intestinalis* demonstrating the highest *in vivo* fitness among the compared species. *In vivo* transcriptome analysis by Eric J.C. Galvez suggested *in vivo* fitness in *Prevotella* species correlates with the number of polysaccharide utilization loci (PULs), with *P. intestinalis* expressing the highest number.

Based on the highest genomic similarity to the predominant human gut *Prevotella* species - *P. copri*, and demonstrated highest *in vivo* fitness, we selected *P. intestinalis* isolate as a representative species to further study the impact of *Prevotella* spp. on the intestinal ecosystem and the host.

4.2.2 *P. intestinalis* reshapes the intestinal microbial community structure

To further study the impact of *P. intestinalis* on the intestinal ecosystem, we colonized specific pathogen free (SPF) WT mice, devoid of any *Prevotella* species, by a single oral gavage of a freshly grown culture. After 4-5 weeks *P. intestinalis* colonization was determined by analyzing fecal microbiota composition using 16S rRNA gene sequencing. Analysis of β-diversity using principle coordinates analysis (PCoA) showed distinct clustering of SPF and SPF+*P. intestinalis* communities (Figure 7A). Based on permutational multivariate analysis of variance (ADONIS), over 60% of the differences were attributed to *Prevotella* colonization (R^2 = 0.62, p<0.001). Strikingly, *P. intestinalis* colonized SPF mice in high relative abundance (50.8% +/- 3.8, mean +/- SEM) (Figure 7B), thereby significantly reshaping the microbial community including a decreased Firmicutes to Bacteroidetes ratio (F/B) (Figure 7C). Although there was no difference in the observed species richness (p=0.26), the complexity of the community structure, when accounting for species richness

and evenness (Shannon index), was significantly lower in SPF mice after *P. intestinalis* colonization (p<0.0001) (Figure 7D). On a family level, comparison of SPF communities with and without *P. intestinalis* colonization by linear discriminant analysis (LDA) effect size (LEfSe) showed that *Prevotella* colonization decreased relative abundance of resident families within the Deferribacteres and Bacteroidetes phyla, as well as most of the Firmicutes (Figure 7E).

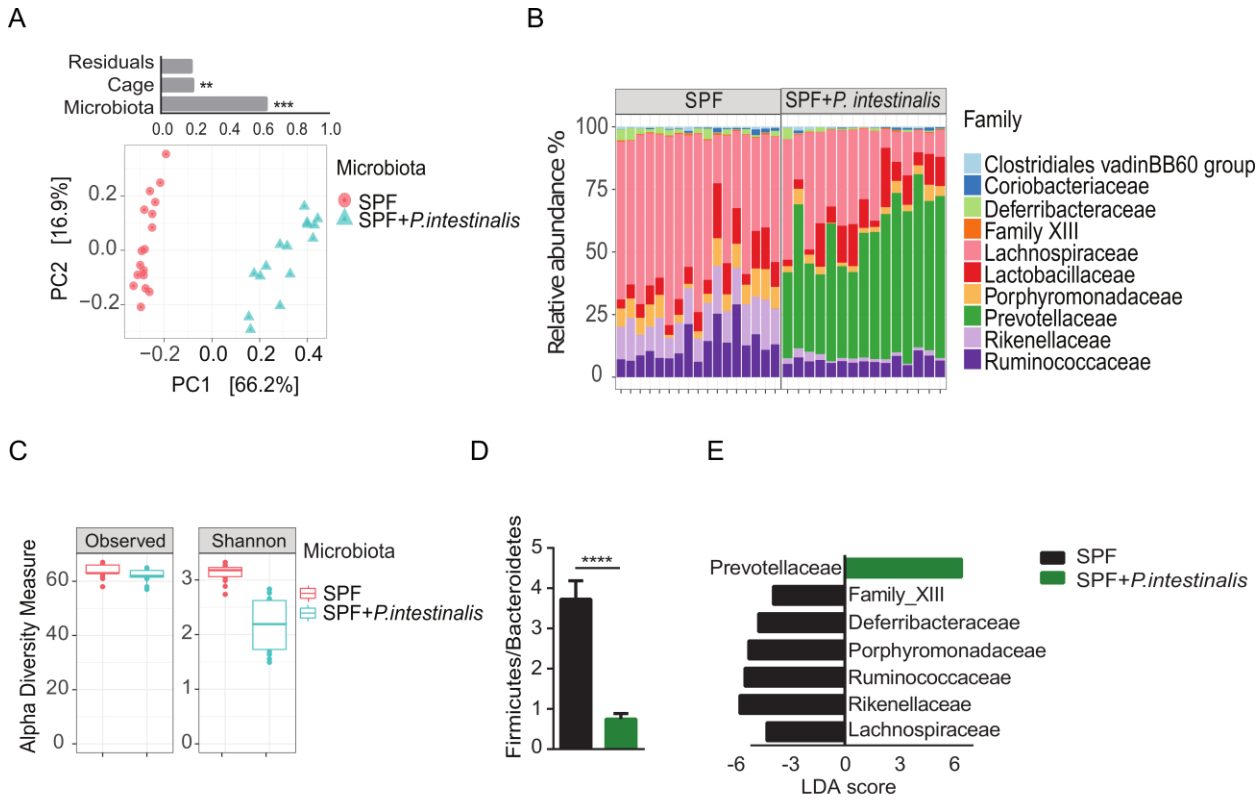


Figure 7: *P. intestinalis* colonization reshapes the resident SPF community

Analysis of β-diversity (PCoA) using Bray-Curtis distances along with multivariate analysis of variance (ADONIS test) of variables 'microbiota, and 'cage' (A) and fecal microbiota composition on the family level using 16S rRNA gene sequencing (B). α-diversity (Observed and Shannon) in SPF and SPF+*P. intestinalis* mice (Student t-test) (C). Ratio of relative abundances between Firmicutes and Bacteroidetes (F/B) (D) and analysis of differentially abundant bacterial families in SPF+*P. intestinalis* microbiota by LEfSe (Kruskal-Wallis test p<0.05, LDA 4.0) (E). The results are shown as mean ± SEM from three independent experiments. P values indicated represent an unpaired nonparametric Mann-Whitney test if not indicated differently *p < 0.05; **p < 0.01; ***p< 0.001; ****p< 0.0001.

4.2.3 Biogeography of *P. intestinalis* colonization

Prevotella spp. have been found to predominantly colonize the lumen of the lower gastrointestinal tract (GIT)[37,210], but have been as well described as a part of the intestinal mucosal community[211,212]. In order to investigate the extent to which the fecal microbiota of SPF+*P. intestinalis* mice reflects the composition of luminal sites, we analyzed the microbial composition in the lumen of distal (DC) and proximal (PC) colon, cecum (CEC), and small intestine (SI) with the focus on *P. intestinalis* abundance (Figure 8A). Comparably to stool samples, *P. intestinalis* was found in high relative abundance in DC (52.3% +/- 4.7, mean +/- SEM), PC (52.5% +/- 5.6, mean +/- SEM), and CEC (37% +/- 1.4, mean +/- SEM). In contrast, *P. intestinalis* was found in significantly lower relative abundance in SI (2% +/- 0.7, mean +/- SEM, p=0.002), likely to reflect the physiological differences within distinct gastrointestinal sites, including pH, oxygen gradient, and antimicrobial components[213].

The combination of 16S rRNA gene sequencing with the quantitative profiling of microbial loads has recently described variation within the absolute abundances of intestinal bacteria and linked it to enterotypes in healthy humans[214]. Hence, we quantified bacterial loads using flow cytometry-based enumeration of bacterial concentrations in the luminal content. This revealed no differences in the total bacterial cell counts after *Prevotella* colonization suggesting that *Prevotella* is not simply increasing the total microbial density, but rather replaces other bacteria (Figure 8B). We additionally analyzed the composition of the mucosa-associated microbiota in DC and PC, locations with highest *P. intestinalis* colonization. We found *P. intestinalis* to be present in both DC and PC mucosal sites, with higher abundance in DC (23.9% +/- 2.6, mean +/- SEM), yet significantly lower than in the DC lumen (Figure 8C).

Altogether, these data demonstrates that *P. intestinalis* colonization has a significant impact on SPF community structure, including the decrease in the microbial diversity and Firmicutes to Bacteroidetes ratio. In addition, *P. intestinalis* predominantly colonizes the lumen of colon and cecum, and can be found closely associated to the colonic mucosa, which is in line with previous findings regarding the niche of *Prevotella* spp. and where it may exert immunomodulatory effects on the host[135].

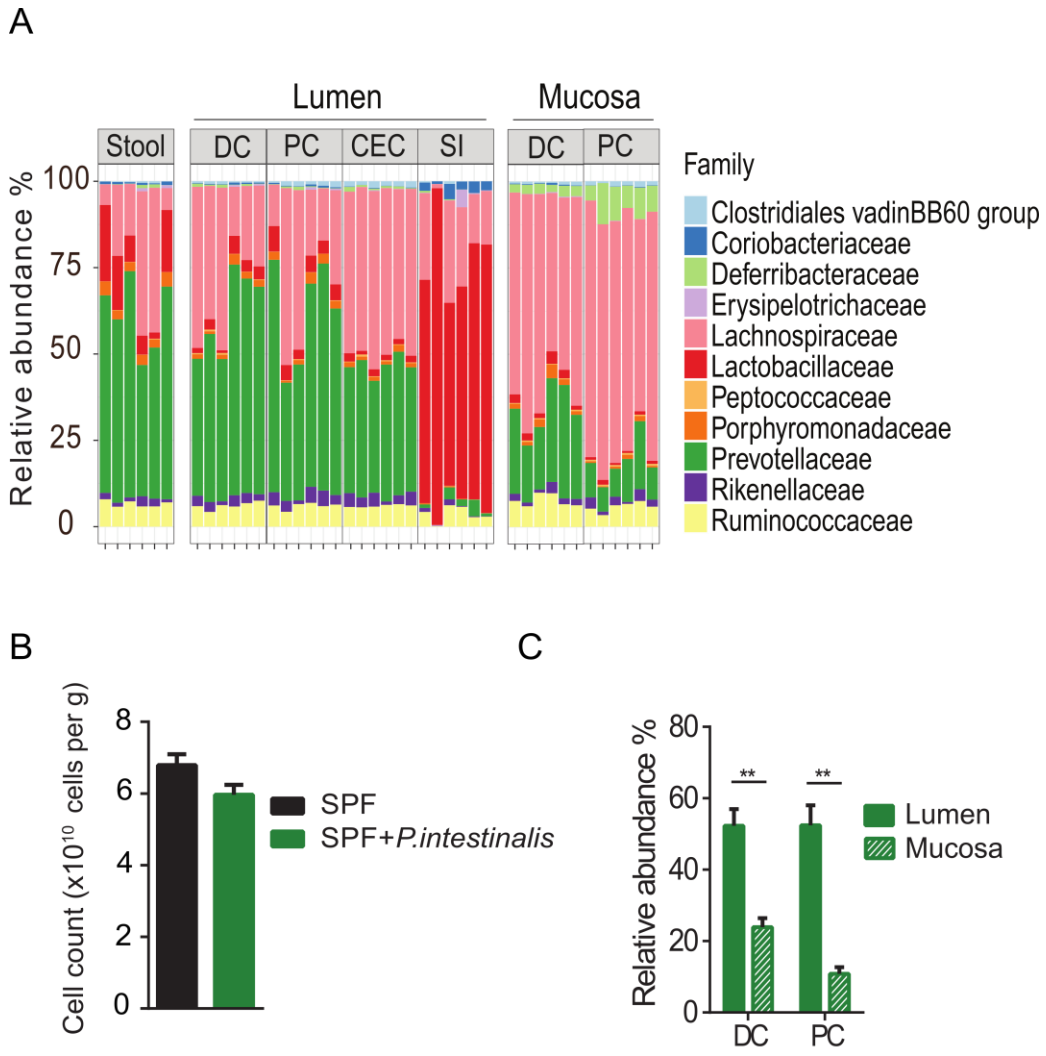


Figure 8: Biogeography of intestinal colonization by *P. intestinalis*

Intestinal microbiota composition in the stool, lumen of distal (DC) and proximal (PC) colon, cecum (CEC), and small intestine (SI), and DC and PC mucosa (A), and flow cytometric enumeration of microbial cells in luminal content (cecum and colon) of SPF and SPF+*P. intestinalis* mice (B). Relative abundance of *P. intestinalis* in the mucosa of DC and PC colon (C). Data shown as mean +/- SEM. *p < 0.05; **p < 0.01 performed with unpaired nonparametric Mann-Whitney test.

4.3 *P. intestinalis* induces minor changes in the host transcriptome in the intestine

Members of the intestinal microbiota are known modulators of the host immune responses which can lead to the development of the inflammatory diseases[67,215]. In order to investigate whether *Prevotella*-induced changes in the microbiota of SPF mice induce significant alterations in the host gene expression, we performed RNA sequencing (RNA-seq) for host transcriptome analysis in the colon of SPF and SPF+*P. intestinalis* mice.

A principal component analysis of gene expression profiles revealed significant difference between SPF and SPF+*P. intestinalis* mice (Figure 9A). However, the analysis of differentially expressed genes (log2 fold change >2.0; $p<0.05$) demonstrated only minor effects of *Prevotella* colonization on the tissue transcriptome (Figure 9B). In *Prevotella*-colonized mice, we identified 35 upregulated and 27 downregulated genes when compared to the SPF untreated mice (Figure 9C). Some of the upregulated genes include genes important for cytokine and chemokine signaling (*IL22ra2, Cxcr5*), B cell development, differentiation, and activation (*Cd19, Cd79b, Tnfrsf13c, Ms4a1*), T cell proliferation (*Tnfrsf13c*). Moreover, *Prevotella* colonization of SPF mice resulted in upregulation of the *Ubd* gene (encoding the Ubiquitin-Like Protein FAT10) expression, which had been reported to be inducible by TNF-α and IFN-γ, and implicated to play a role in numerous cellular processes, including NF-kB activation and caspase-dependent cell death[216–218]. Surprisingly, several genes upregulated in *Prevotella*-colonized mice are known to be expressed in a circadian pattern and form a core component of the circadian clock (*Per2, Per3, Ciart*).

In addition, pathway enrichment analysis based on gene ontology (GO) showed that many upregulated genes in *Prevotella*-colonized mice were involved “Immune system processes” and “B cell activation” (Figure 9D). These data suggests *P. intestinalis* may have immuno-modulatory effects on the host, however further experimental validation is necessary to substantiate the specific findings. Whether *P. intestinalis* colonization indeed activates B cells or impacts T cell proliferation will be assessed by global immunophenotyping.

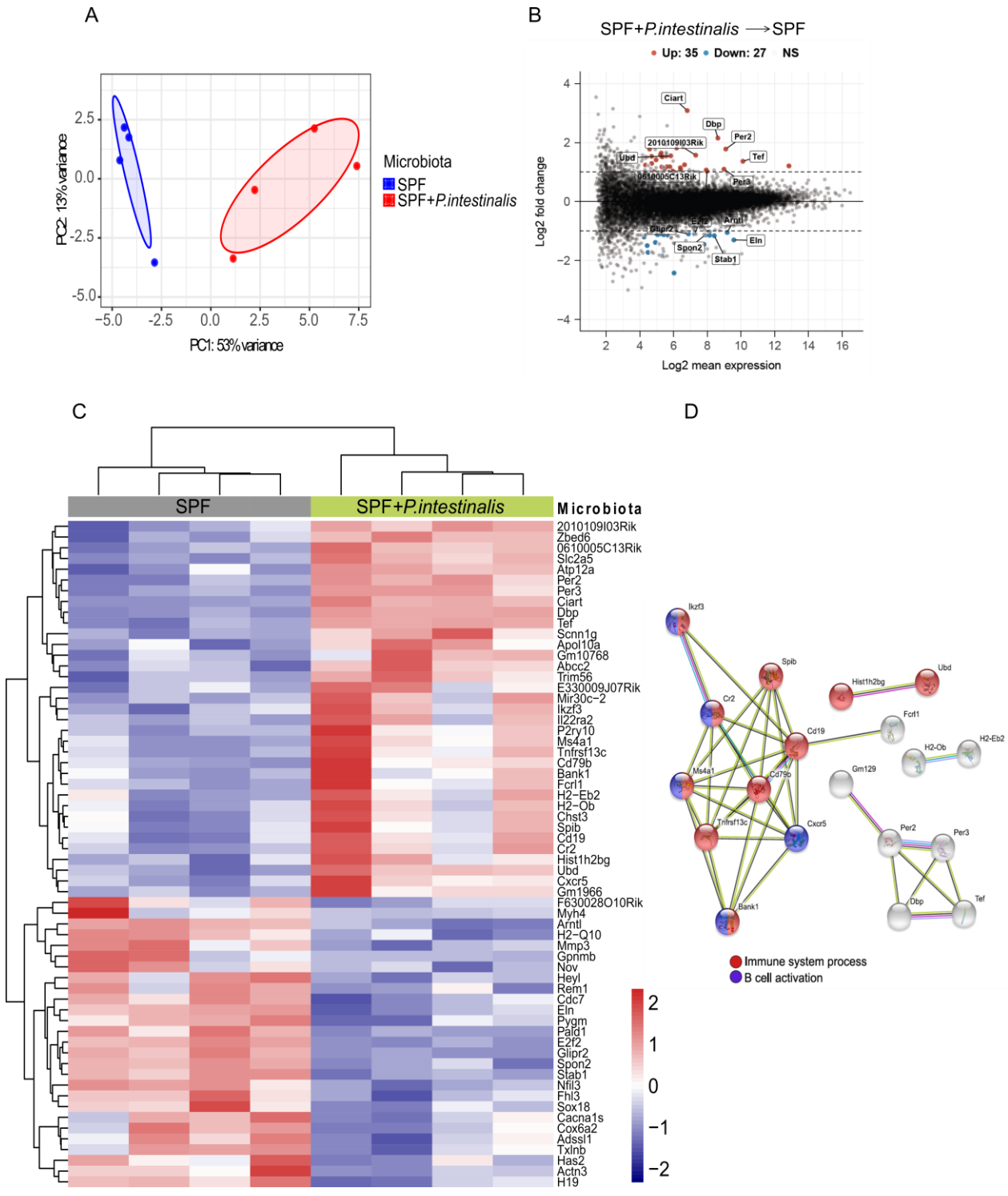


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Figure 9: *Prevotella*-induced host transcriptome changes in colon tissue of SPF and SPF+*P. intestinalis* mice

SPF mice were colonized with *P. intestinalis* for 5 weeks and host transcriptome analysis was performed from distal colon tissue of SPF and *P. intestinalis* mice. Principal component analysis of gene expression profile by RNAseq (A). Log ratio and mean average plot (MA-plot,). Gene expression levels in non-colonized SPF mice vs. SPF colonized with *P. intestinalis*. Colored dots indicate genes with log2 fold change >2.0; p<0.05. RED – upregulated in SPF+*P. intestinalis*, BLUE - upregulated in SPF control (B). Heatmap displays the read counts of significant differentially expressed genes (C). Pathway enrichment analysis based on gene ontology (GO) using upregulated genes in SPF+*P. intestinalis* (D).

4.4 *P. intestinalis* colonization exacerbates intestinal inflammation

Increased abundance of *P. intestinalis*, at that time described as an unknown and uncultured member of the intestinal microbiota in NLRP6-deficient mice, was associated with higher susceptibility to chemically-induced colitis[18]. In addition, presence of *Prevotella* species was described in distinct lines of immunocompetent mice prone to intestinal inflammation[67]. While these effects were not causally linked to the presence of *Prevotella*, the data suggested that a *Prevotella*-dominated microbiome may have the propensity to promote inflammation and intestinal dysbiosis.

4.4.1 *P. intestinalis* colonization alters susceptibility to DSS-induced colitis in immunocompetent host

We next wanted to investigate whether *P. intestinalis* colonization by *P. intestinalis* of SPF community is sufficient to alter the susceptibility to intestinal inflammation after induced damage to the intestinal barrier. Notably, WT SPF mice used in this study have been previously reported to be relatively resistant to induction of DSS colitis, displaying moderate colitis severity and mild weight loss[67]. Therefore, we colonized 5 weeks old WT SPF mice with a single oral gavage of *P. intestinalis* culture and, after 5 weeks of colonization, acute intestinal inflammation was induced by administering dextran sulfate sodium (DSS) in drinking water (2.1% w/v). Colonization of SPF mice with *P. intestinalis* resulted in more

severe disease outcome as displayed by the significant increased body weight loss when compared to control SPF mice (Figure 10A). However, mice colonized with *P. intestinalis* did not show an increase in mortality (Figure 10B). Colonoscopy on days 6 and 9 after induction of DSS colitis revealed increased tissue damage in *P. intestinalis* colonized mice (Figure 10C and 10D).

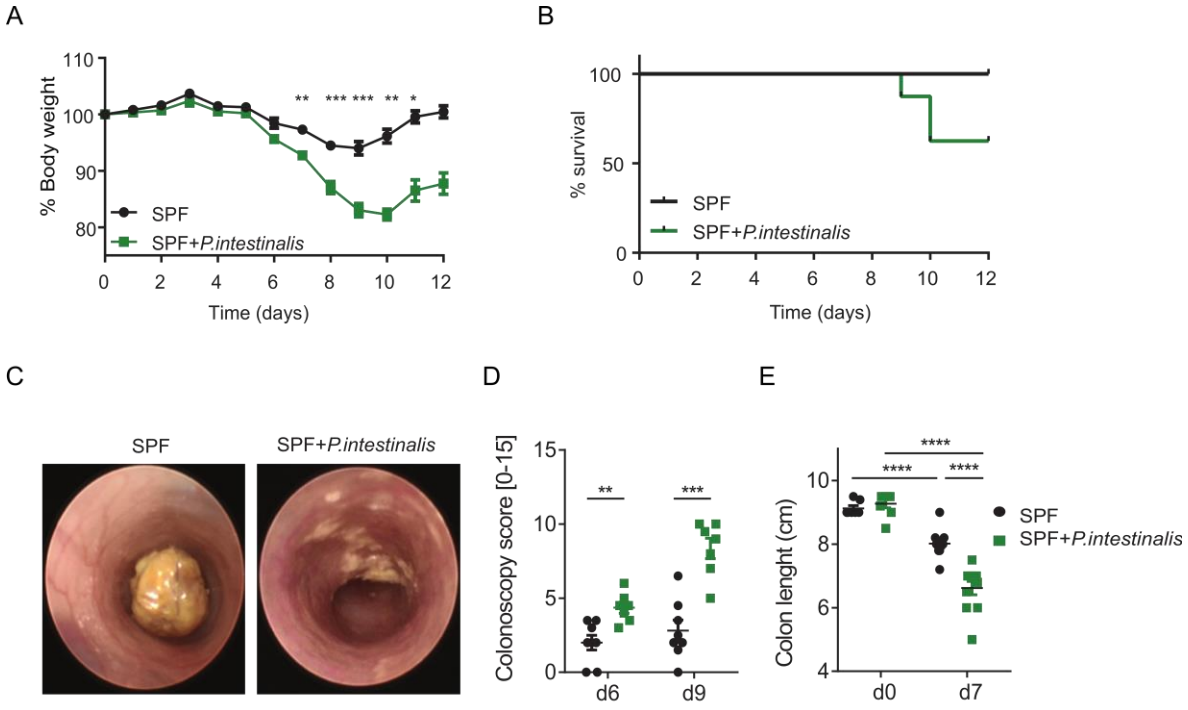

Figure 10: *P. intestinalis* colonization alters the colitis susceptibility of WT SPF mice

Body weight loss (A) and survival (B) of WT mice harboring SPF and SPF+*P. intestinalis* microbiota during DSS colitis (2.1 % w/v for 7 days). Representative colonoscopy images of colitis severity (C) performed on day 6 after colitis induction in SPF and SPF+*P. intestinalis* mice and colitis score (D) on day 6 and day 9 of DSS colitis, based on five parameters: granularity of mucosal surface, vascular pattern, translucency of the colon mucosa, visible fibrin and stool consistency. Colon length (E) during steady state (d0) and DSS (d7). The results are shown as mean +/- SEM. *p < 0.05; **p < 0.01***p< 0.001; ****p< 0.0001 performed with unpaired nonparametric Mann-Whitney test.

Moreover, higher intestinal inflammation of *Prevotella*-colonized mice was supported by pronounced colon shortening (Figure 10E) and histological characterization of tissue damage during DSS colitis (Figure 11). Whereas the histological analysis of cecum and small intestine during DSS colitis showed no significant differences between SPF and SPF+*P. intestinalis* mice (Figure 11A and 11B), *Prevotella* colonization induced significant

inflammation in the colon (Figure 11C). Inflammation in *P. intestinalis* colonized mice was highest in the distal colon with pronounced tissue erosion, higher hyperplasia, edema, and infiltration of inflammatory cells (Figure 11D and 11E). Notably, histological analysis of colonic tissue between SPF and *Prevotella*-colonized mice revealed no differences during steady state (Figure 11D and 11E). Together, these data demonstrate that *P. intestinalis* is able to alter susceptibility to DSS colitis even in an immunocompetent host.

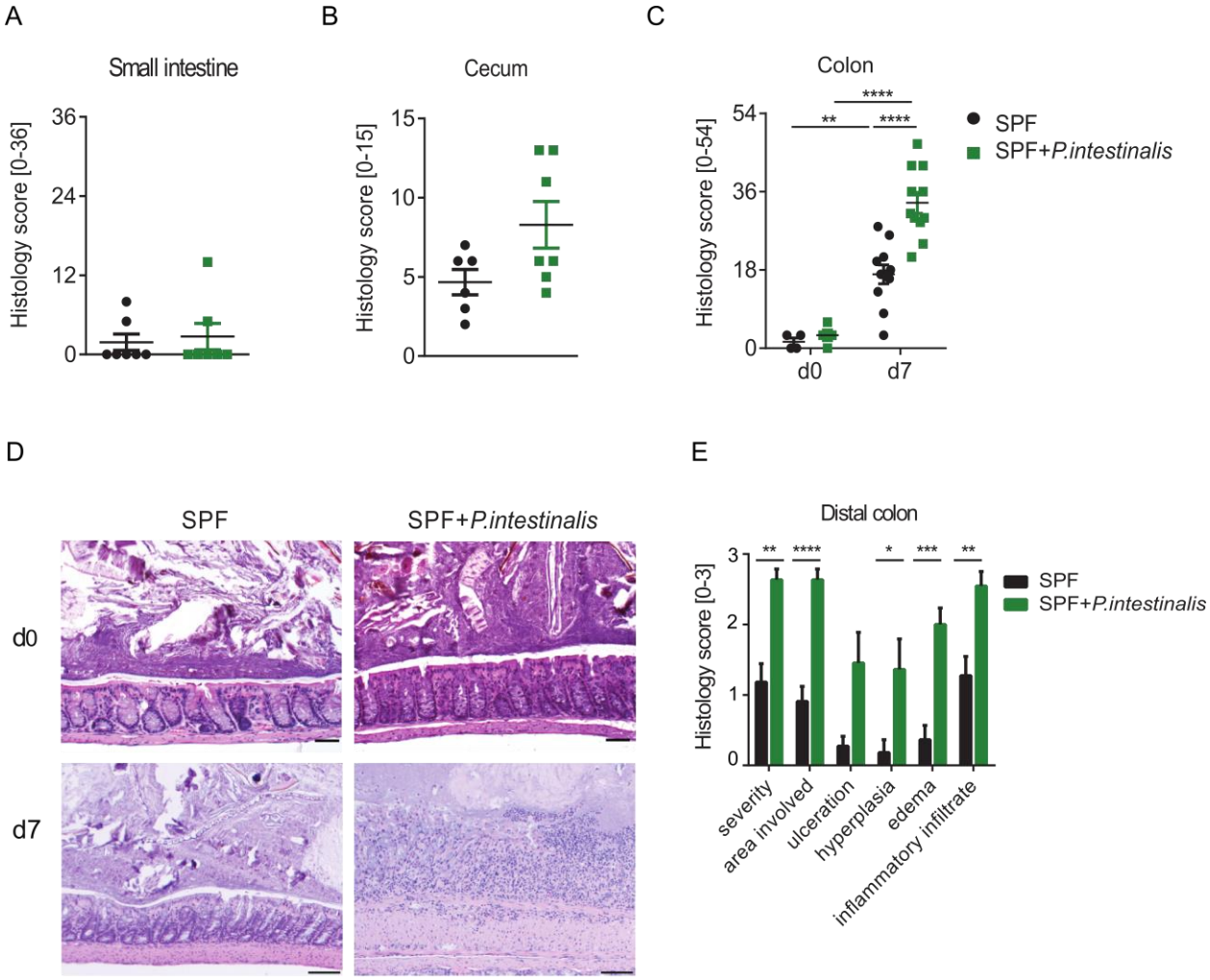

Figure 11: Histological evaluation of colitis severity in SPF and SPF+P. intestinalis mice. Histological evaluation of small intestine (A), cecum (B), and colon (C) in SPF and SPF+*P.intestinalis* mice measured during steady state (d0) in colon and during DSS colitis (d7). Representative images of H&E-stained distal colon sections on d0 and d7 of DSS colitis (D), and histological analysis of d7 distal colon sections by each scoring parameter (E). The results are shown as mean +/- SEM. *p < 0.05; **p < 0.01***p< 0.001; ****p< 0.0001 performed with unpaired nonparametric Mann-Whitney test.

4.4.2 Altered DSS susceptibility by *P. intestinalis* colonization is associated with elevated pro-inflammatory cytokine responses

To further characterize the differences in inflammation between SPF and SPF+*P. intestinalis* mice, various cytokines and chemokines were quantified in the distal colon tissue in steady state and during inflammation. Levels of the pro-inflammatory cytokines IL-6 and tumor necrosis factor alpha (TNF-α) were higher in mice harboring *P. intestinalis* (Figure 12) on day 7 of DSS colitis. *P. intestinalis* colonization also resulted in increased levels of the anti-inflammatory cytokine IL-10 in the colon. Notably, contrary to the results observed in SPF mice colonized with the *Prevotella*-rich microbial community from *Nlrp6*$^{-/-}$ mice from which *P. intestinalis* was originally isolated[67], mice colonized with *P. intestinalis* alone did not display increase in levels of interferon (IFN)- γ, IL-17A, IL-1β, or CCL5 (Figure 12).

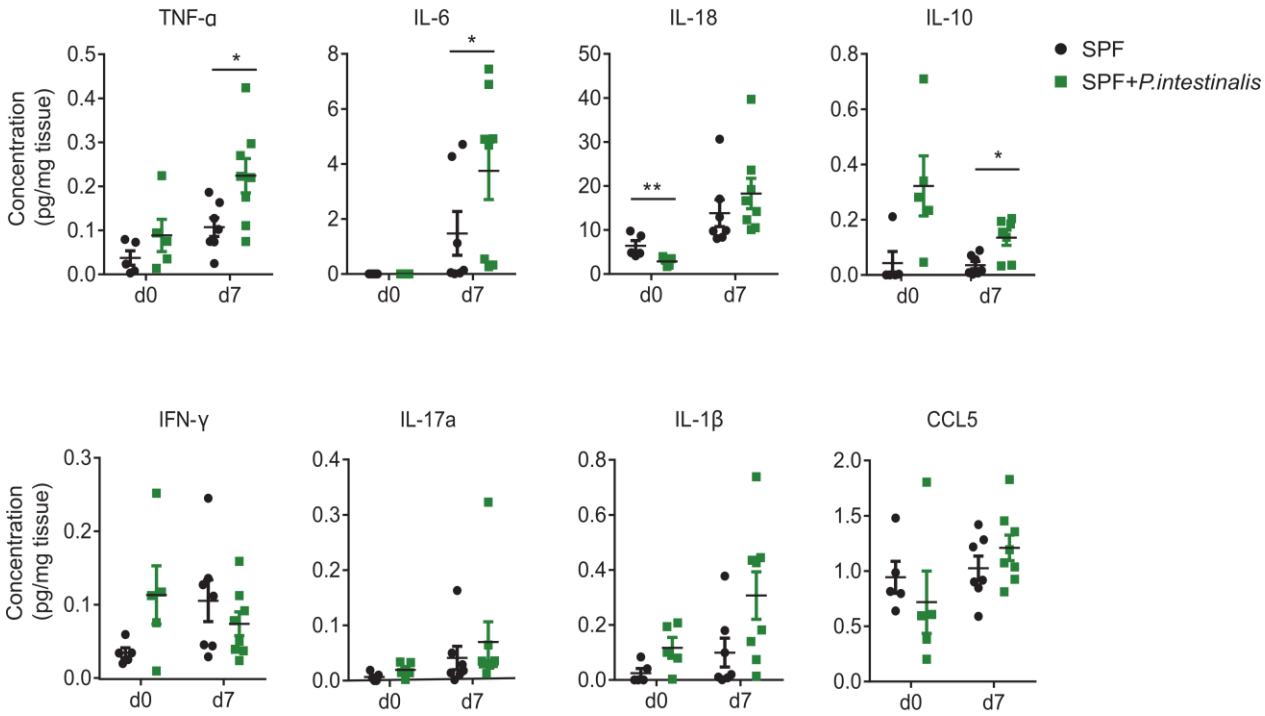

Figure 12: Production of pro-inflammatory cytokines during steady state and DSS colitis in SPF and *Prevotella*-colonized mice

Concentration of cytokines measured from distal colon tissue homogenates of SPF and SPF+*P. intestinalis* mice on day 0 and day 7 of DSS, analyzed using LEGENDplex kit or ELISA (IL-18). Data represented as mean +/- SEM. *p < 0.05; **p < 0.01 performed with unpaired nonparametric Mann-Whitney test.

Strikingly, there was no impact of *P. intestinalis* colonization on levels of a range of cytokines tested during the steady state when compared to the SPF mice, except a decrease of IL-18 levels in distal colons of *Prevotella*-colonized mice. Higher intestinal inflammation in SPF+*P. intestinalis* mice during DSS colitis was also characterized by significant increase of multiple chemokines, including LIX and MCP-1, which have been involved in the recruitment and activation of monocyte and neutrophils to the site of inflammation, as well as MIP-1α and MIP-1β (Figure 13).

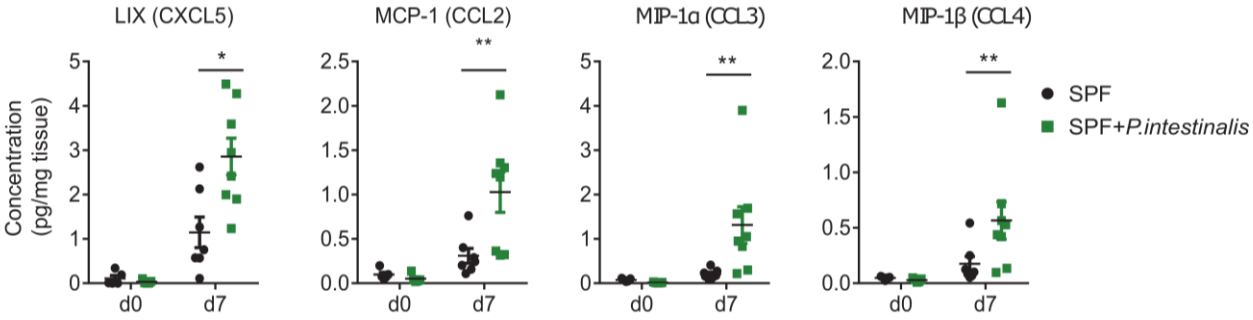

Figure 13: Production of pro-inflammatory chemokines during steady state and DSS colitis in SPF and *Prevotella*-colonized mice

Concentration of chemokines measured from distal colon tissue homogenates of SPF and SPF+*P. intestinalis* mice on day 0 and day 7 of DSS, analyzed using LEGENDplex kit or ELISA (IL-18). Data shown as mean +/- SEM. *p < 0.05; **p < 0.01 performed with unpaired nonparametric Mann-Whitney test.

4.4.3 *Prevotella*-induced inflammation is associated with elevated neutrophil infiltration

Inflammation in DSS colitis can be triggered by different effector cells including innate and adaptive immune cells[67,219]. To identify which subsets of immune cells are differently present between the two groups, we analyzed the abundance and composition of colonic lamina propria leukocytes (cLPLs) before and 7 days after induction of DSS colitis by flow cytometry. The analysis of the innate cLPLs (Figure 14A) showed that *Prevotella* colonization resulted in increased frequency and numbers of LPLs (CD45+ cells) after the DSS induction, but no difference was observed between SPF and *Prevotella*-colonized mice during the steady state (Figure 14B and 14C). Analyzing the abundance of various subsets

of innate immune cells, we observed a significant increase in frequency and numbers of neutrophils (Ly6C+Ly6G+ cells) in colons of mice colonized with *P. intestinalis* during DSS colitis, but not in the steady state (Figure 14D-F). These findings are in line with the increased levels of multiple neutrophil-attracting chemokines we measured in colons of SPF+*P. intestinalis* mice (Figure 13A). We observed no significant increases in MHCII+CD11b+CD11c- or MHCII+CD11c+CD11b+/- cells, containing both macrophages and dendritic cells (Figure 14G).

The global analysis of immune cells subsets of the adaptive immune system in colon tissue demonstrated no significant differences in cell numbers or frequencies (Figure 15A), i.e. we observed no differences in the numbers and abundances of total CD4+ and CD8+ T cells (Figure 15B-C) as well as B220+ B cells (not shown). Notably, while the numbers and frequency of activated CD4+ T cells (CD62L-CD44+) were increased in colons of *Prevotella*-colonized mice during DSS colitis (Figure 15D and 15E), the numbers of different CD4+ T helper (Th) subsets including Th1 (CD4+IFN-γ+) and Th17 (CD4+IL-17+) cells as well as in regulatory T cells (CD4+Foxp3+) were not affected (Figure 15F-H). These data suggest that exacerbation of DSS colitis severity by *P. intestinalis* is associated with differential recruitment and activation of innate and to a lesser degree of adaptive immune cells, respectively.

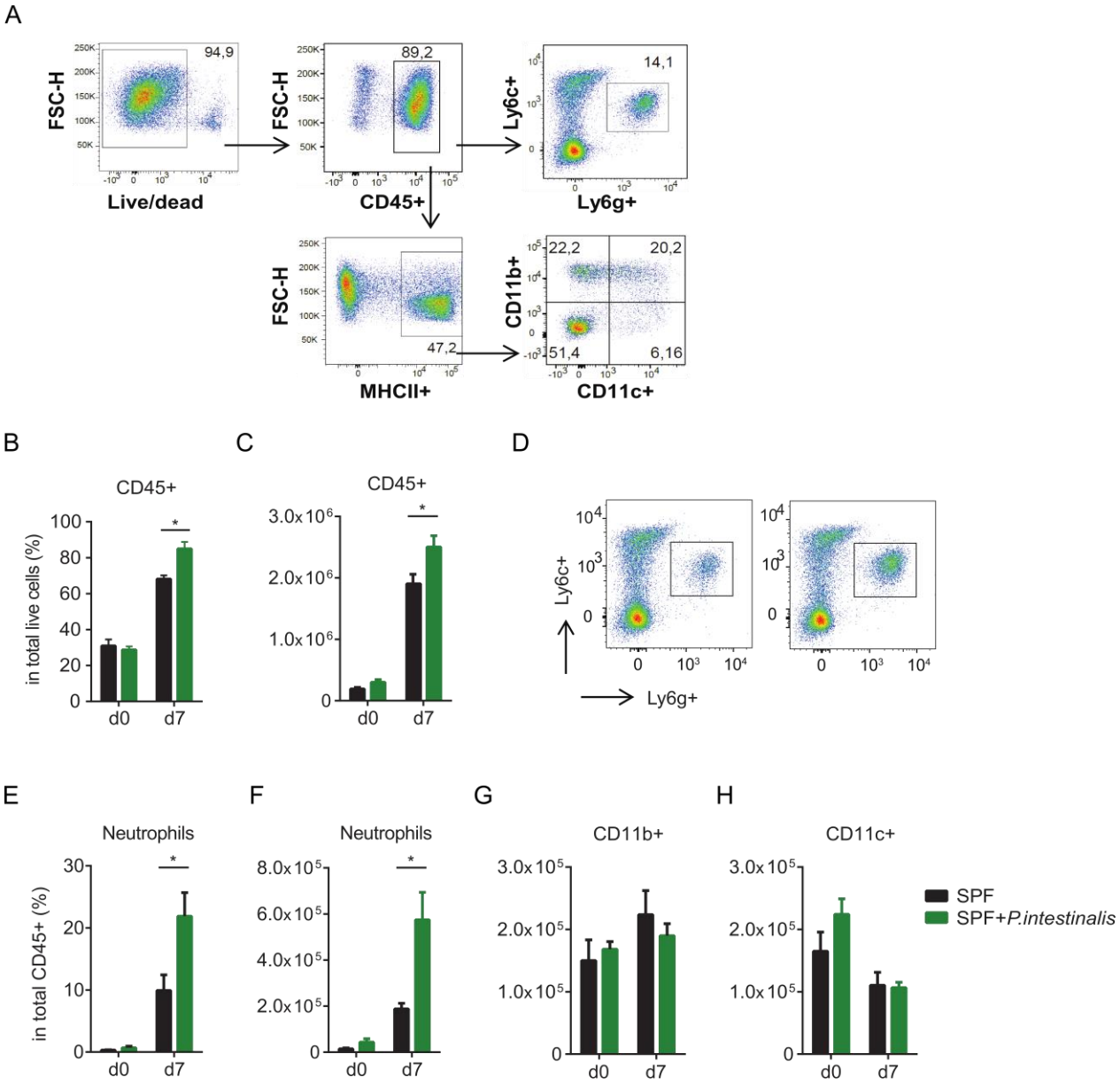

Figure 14: Analysis of innate colonic LPLs in SPF and SPF+*P. intestinalis* mice

Colonic lamina propria leukocytes (cLPLs) were isolated from WT mice harboring SPF and SPF+*P. intestinalis* microbiota, during the steady state (d0) and on day 7 (d7) during DSS colitis, and analyzed by fluorescence-activated cell sorting (FACS). Gating strategy of FACS data is displayed for panel of innate cells (A). Frequency (B) and total number of CD45+ cells in cLPLs (C). Representative FACS plots of analysis of neutrophil infiltration upon DSS induction (d7) (D), and frequencies (E) and total numbers (F) of neutrophils on d0 and d7 DSS. Total cell numbers of CD11b+ (G) and CD11c+ (H) cells. The results are shown as mean +/- SEM. *p < 0.05 performed with unpaired nonparametric Mann-Whitney test.

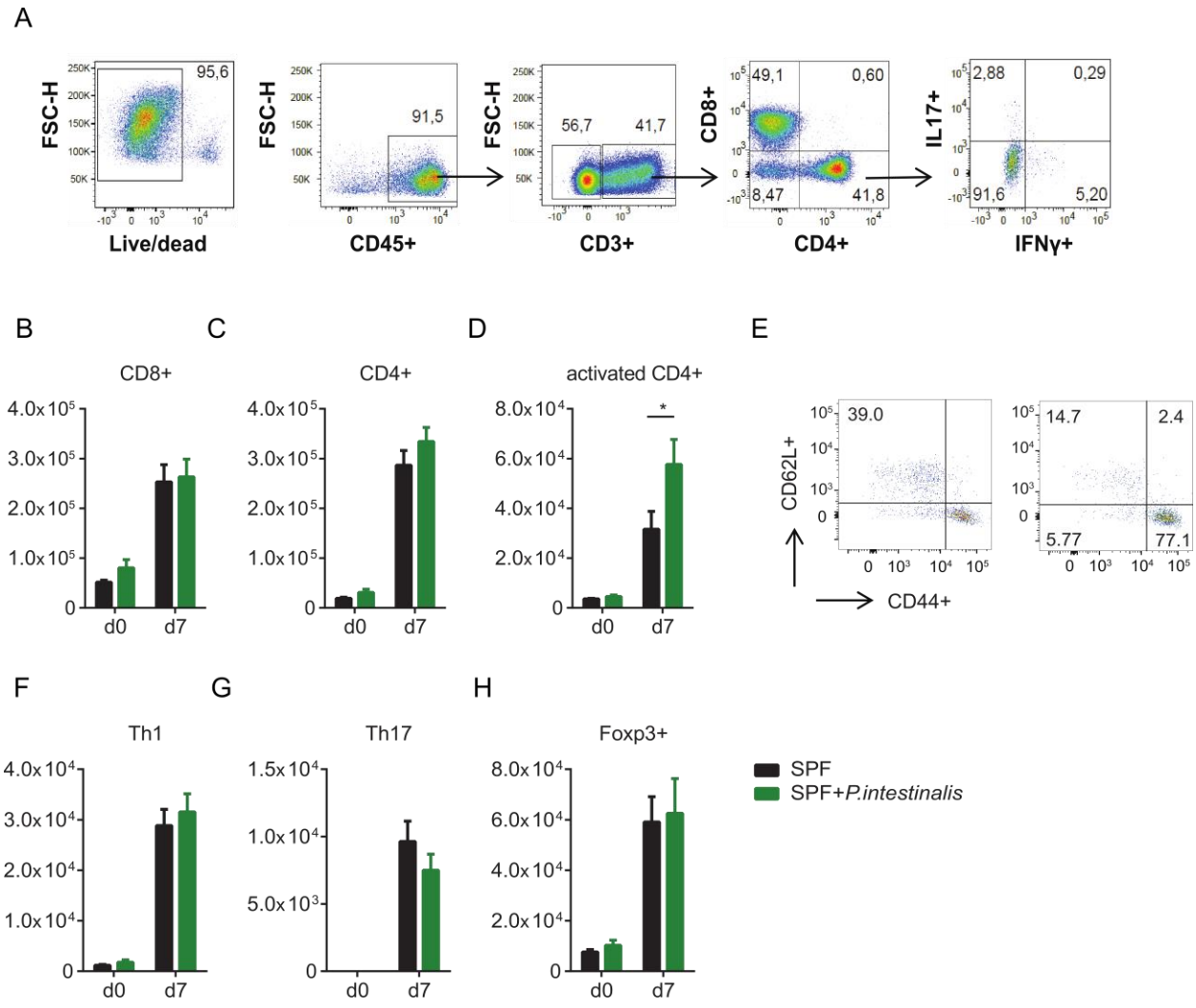

Figure 15: Analysis of T cell subsets in SPF and SPF+*P. intestinalis* mice in steady state and DSS-induced colitis

Colonic lamina propria leukocytes were isolated from WT mice harboring SPF and SPF+*P. intestinalis* microbiota, during the steady state (d0) and on day 7 (d7) during DSS colitis, and analyzed by FACS. Gating strategy of FACS data are displayed for panel of adaptive cells (A). Total number of CD8+ (B), CD4+ (C), and activated (CD44+) T cells (D) in cLPLs. Representative FACS plots of analysis of activated T cells on day 7 during DSS (E), and total numbers of subsets of helper T cells (F-H) on d0 and d7 DSS. Data shown as mean +/- SEM. *p < 0.05 performed with unpaired nonparametric Mann-Whitney test.

4.4.4 *Prevotella*-induced inflammation is independent of adaptive immunity

We recently demonstrated that the colitogenic community of *Nlrp6*$^{-/-}$ mice, which contains *P. intestinalis*, alters susceptibility to DSS colitis via modulation of adaptive immune cells, i.e. the transfer of the community in *Rag2*$^{-/-}$ mice was unable to exacerbate disease severity[67]. To test whether *P. intestinalis* requires the presence of adaptive immune cells to alter colitis susceptibility, we colonized WT and Rag2-deficient mice with *P. intestinalis*. Importantly, both WT and *Rag2*$^{-/-}$ mice harbored the same SPF microbiota before the *P. intestinalis* colonization (Figure 16A and 16B). Specifically, the comparison of their fecal microbiota composition before induction of DSS colitis showed that the mice clustered together in relation to their microbial communities (SPF or SPF+*P. intestinalis*) (Figure 16B). Multivariate analysis of variance using ADONIS showed microbiota contributed to the variability of the groups with 60% (R^2=0.60, p=0.001), while genotype contributing to the differences as little as 5% (R^2=0.05, p=0.007). Strikingly, *P. intestinalis* exacerbated DSS colitis severity both in WT and *Rag2*$^{-/-}$ mice, as indicated by their weight loss (Figure 16C) and colon shortening (Figure 16D). Taken together, these results show that *P. intestinalis* colonization promotes intestinal inflammation upon damage to the intestinal barrier independent of adaptive immune cells.

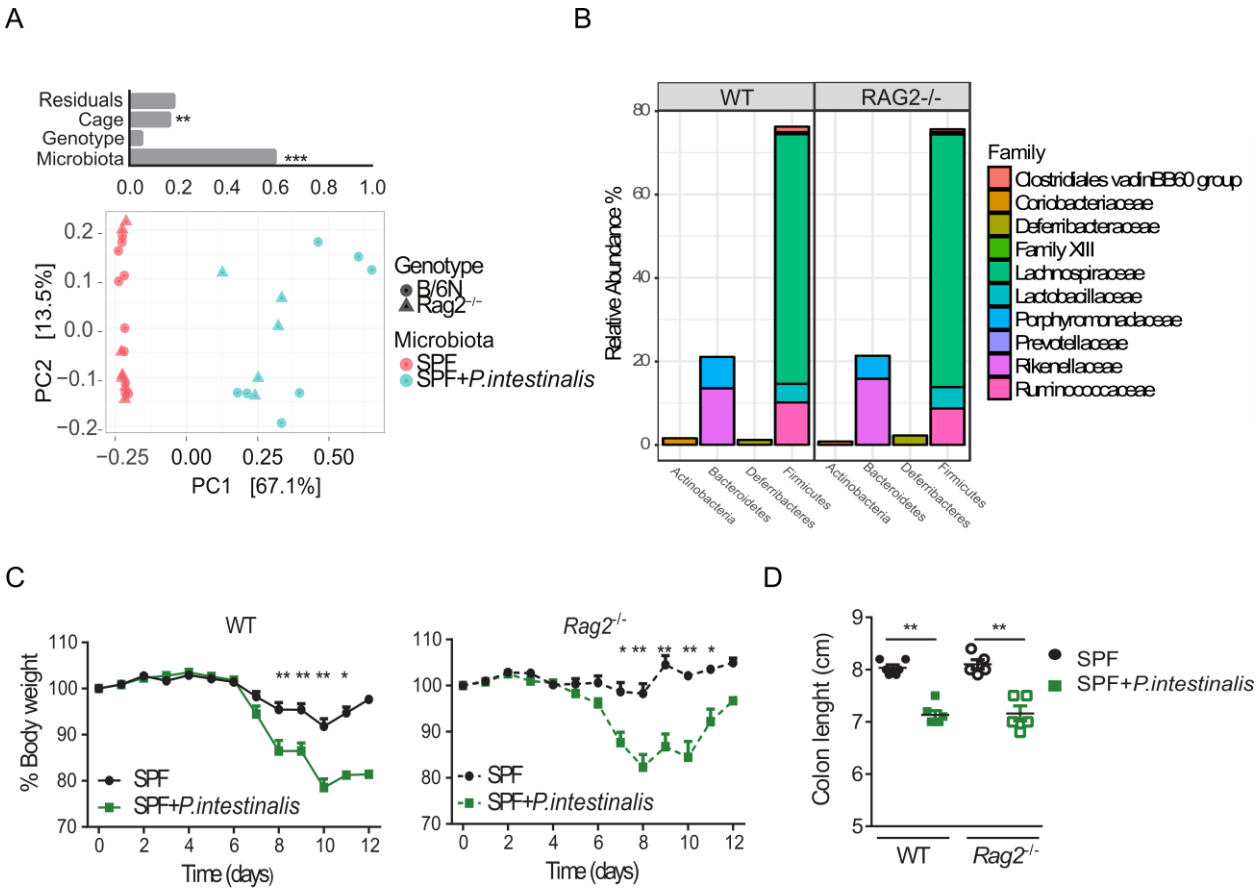

Figure 16: *Prevotella*-exacerbated intestinal inflammation is independent of adaptive immune system

WT and *Rag2*$^{-/-}$ mice with SPF microbiota were left untreated or colonized with *P. intestinalis* for 5 week before induction of DSS colitis Analysis of β-diversity (PCoA) of WT and *Rag2*$^{-/-}$ harboring SPF and SPF+ *P. intestinalis* mice along with multivariate analysis of variance (ADONIS test) of variables 'microbiota', 'genotype' and 'cage' (A) and fecal microbiota composition analysis of SPF WT and *Rag2*$^{-/-}$ mice on the family level using 16S rRNA gene sequencing (B). Body weight loss during DSS colitis (2.1 % for 7 days) (C) and colon length on day 7 (D). Data shown as mean +/- SEM. *$p < 0.05$; **$p < 0.01$ performed with unpaired nonparametric Mann-Whitney test if not indicated differently.

4.4.5 Alteration of the microbiota composition during DSS colitis

Induction of DSS colitis has been shown to alter the composition of the intestinal microbiota[220]. To analyze the impact of induction of DSS colitis on the microbiota composition of SPF and SPF+*P. intestinalis* mice, and investigate whether intestinal inflammation further promotes *Prevotella* abundance in the gut, we analyzed the microbiota composition before and during DSS colitis. Comparison of fecal microbial communities in SPF and SPF+*P. intestinalis* mice before (d0) and after induction of DSS colitis (d6) reveled significant changes due to induction of DSS colitis (Figure 17A and 17B). The permutational multivariate analysis of variance (ADONIS) showed that 43% of the differences were due to induction of inflammation by DSS treatment ($R^2 = 0.43$, $p<0.001$). Microbiota composition of SPF and *Prevotella*-colonized mice during DSS colitis was characterized by significant decrease of α-diversity, with decrease in specie richness and evenness (Figure 17C). We used the linear discriminant analysis effect size (LEfSe) method to identify the most differentially abundant bacterial families between steady state and during induction of DSS in SPF+*P. intestinalis* mice. Even though we have not observed significant change in *Prevotella* abundance between steady state and during DSS colitis, relative abundance of other members of the Erysipelotrichaceae, Porphyromonadaceae, Lactobacillaceae, and Ruminococcaceae families were increased during inflammation (Figure 17D). In order to determine whether increase in abundance of these bacterial families contributes to the higher susceptibility to DSS colitis we compared differentially abundant families between the SPF and SPF+*P. intestinalis* mice during inflammation. The LEfSe analysis revealed Prevotellaceae as the only differentially abundant member (Figure 17E), whereas other members showed no significant differences and comparable relative abundance in both SPF and SPF+*P. intestinalis* mice. These data suggest that *Prevotella* colonization, rather than the increase in abundance of resident members of the SPF microbial community, is responsible for increased susceptibility to intestinal inflammation.

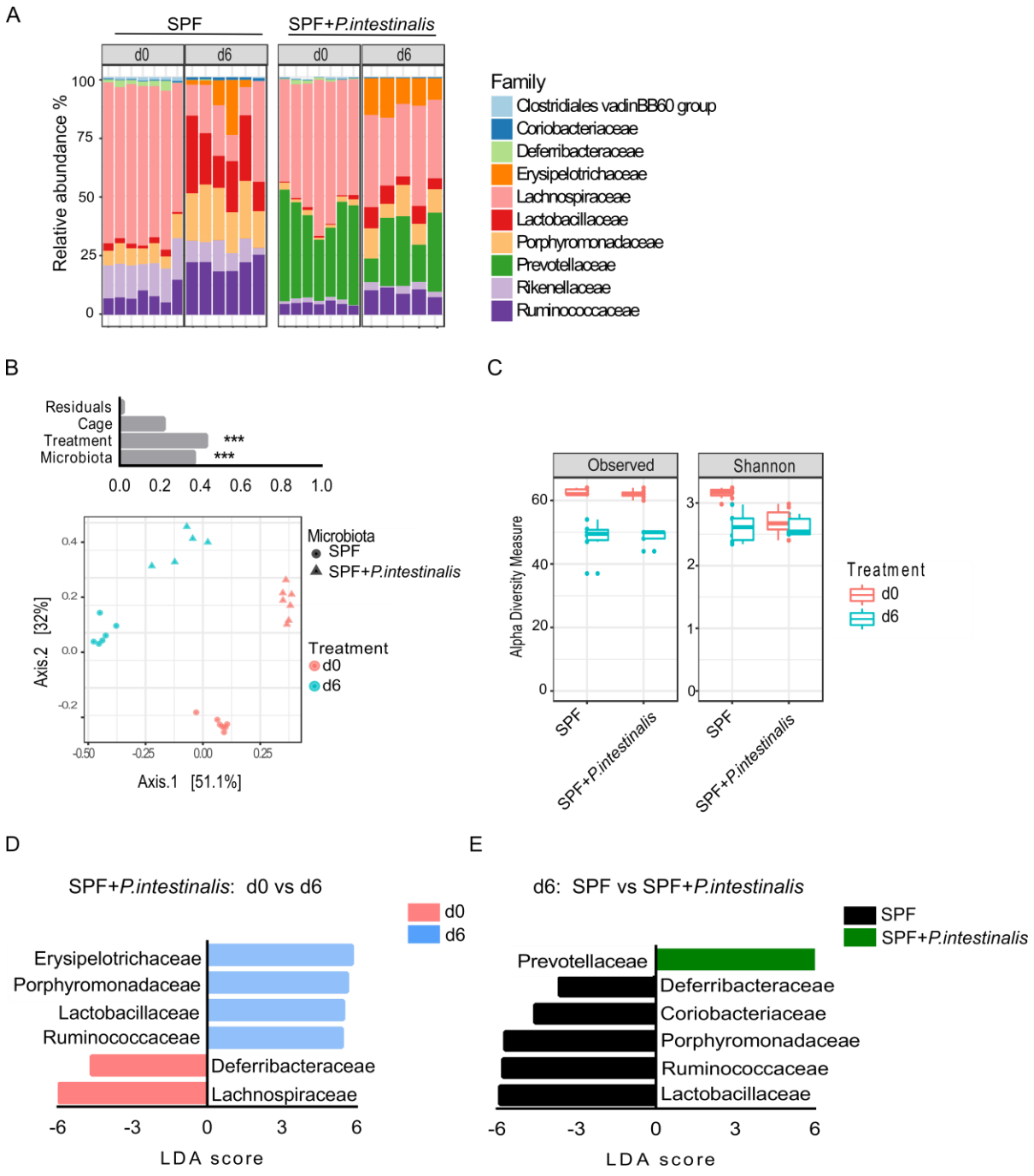

Figure 17: DSS-induced changes in the microbiota of *Prevotella*-colonized mice

Analysis of β-diversity (PCoA) along with multivariate analysis of variance (ADONIS test, ***p < 0.001.) along with multivariate analysis of variance (ADONIS test) of variables 'microbiota', 'treatment' and 'cage' (A), fecal microbiota composition on the family level using 16S rRNA gene sequencing (B), and α-diversity (C) in SPF and SPF+*P. intestinalis* mice during steady state (d0) and DSS-colitis (d6). LEfSe analysis of differentially abundant families between d0 and d6 of DSS colitis on microbiota of SPF+*P. intestinalis* mice (D) and between SPF and SPF+*P. intestinalis* mice on d6 of DSS colitis (E) (Kruskal-Wallis test p<0.05, LDA 4.0).

4.5 Investigation of *P. intestinalis* immunogenic properties

We have shown that *P. intestinalis* colonization alters SPF intestinal community and increases its susceptibility to intestinal inflammation. Whether *P. intestinalis* colonization remodels the microbial community of SPF mice to a more pro-inflammatory composition or increased susceptibility to intestinal inflammation is rather attributed to higher immunogenicity and immuno-stimulatory potential of *P. intestinalis,* remained to be investigated.

4.5.1 *P. intestinalis* monocolonization of germ-free mice

We next wanted to determine *P. intestinalis* immuno-stimulatory potential by using germ-free (GF) mice and excluding the effect of the microbiota alteration. We therefore monocolonized GF mice with *P. intestinalis* and tested for colonization in GF and control SPF mice 7 days after oral gavage (Figure 18A). Surprisingly, no colonization by *P. intestinalis* could be detected in GF mice by *Prevotella*-specific PCR, whereas control SPF mice were colonized as expected (Figure 18B). Further examination of colon sections of GF and SPF mice colonized by *Prevotella* by electron microscopy showed absence of bacteria in both groups (Figure 18C) suggesting that *P. intestinalis* is not able to colonize GF mice. Whether inability to colonize GF mice is a result of lack of microbial cooperation and cross-feeding on nutrients produced by other members of the microbiota, or unfavorable physical and/or chemical properties in GF intestine, such as increase of oxygen levels, remained unclear.

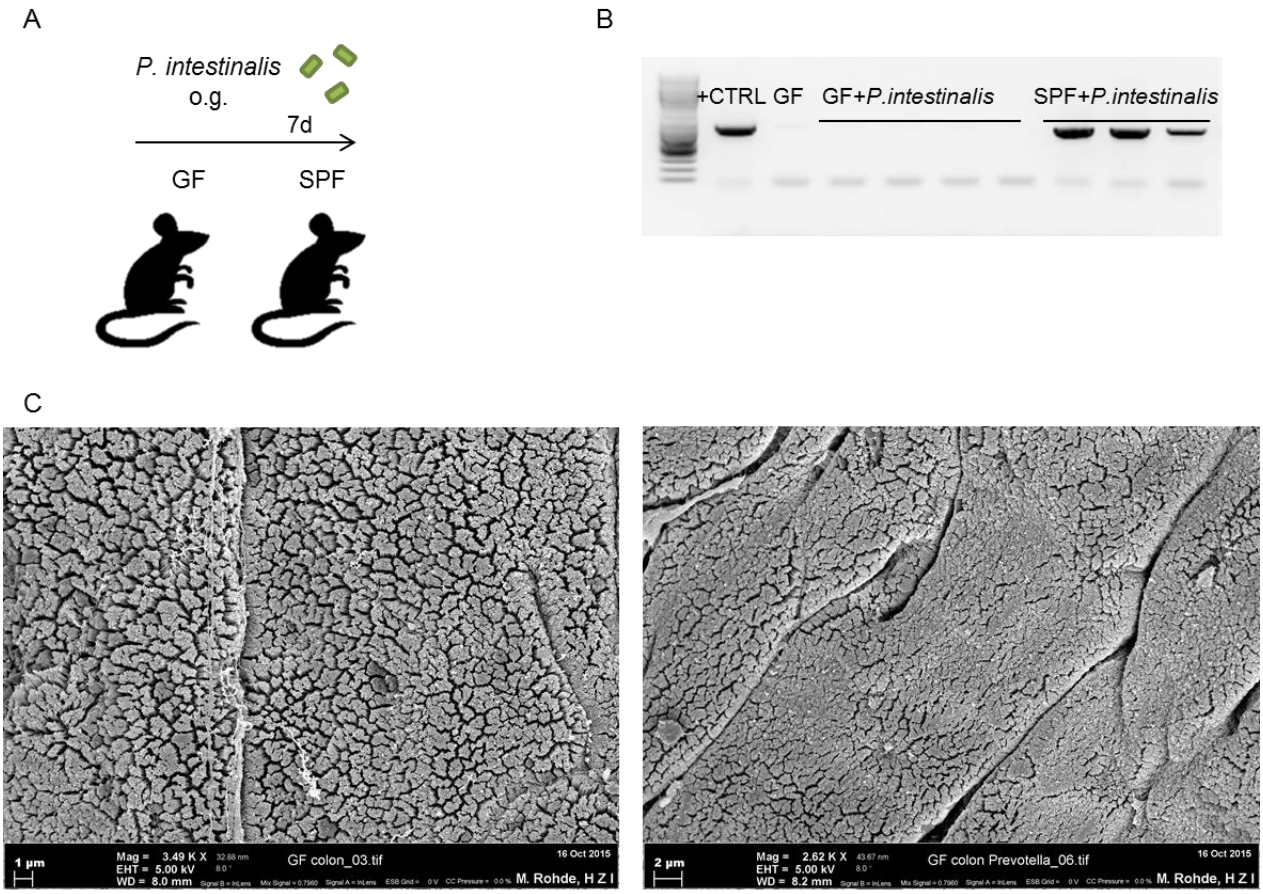

Figure 18: Monocolonization of germ-free mice by *P. intestinalis*

GF and SPF mice were colonized by oral gavage with *P. intestinalis* for 7 days (A) and colonization was determined by *Prevotella*-specific PCR from fecal DNA (B). Electron microscopy images of colon sections in GF (left) and GF+*P. intestinalis* mice (right) (C).

4.5.2 TLR4 senses *P. intestinalis* and triggers a pro-inflammatory immune response *in vitro*

To answer the question whether *P. intestinalis* colonization remodels the microbial community of SPF mice to a more pro-inflammatory composition or directly stimulates the immune response, we stimulated bone marrow-derived macrophages (BMDM) *in vitro* with fecal content from SPF and SPF+*P. intestinalis* mice. Fecal contents were divided in two fractions, microbial community (pellet) and the secreted microbial factors (supernatant), and their potential to trigger IL-6 secretion in BMDM was determined. Strikingly, stimulation of WT BMDM with SPF+*P. intestinalis* microbial community resulted in higher levels of pro-inflammatory response in comparison to SPF community alone (Figure 19A). Secreted

microbial factors stimulated the macrophages to a lesser degree and with no significant differences between the SPF and SPF+ *P. intestinalis* communities (Figure 19B). These data corroborate the *in vivo* effects of *P. intestinalis* on colonic inflammation during DSS colitis, and suggest that cell-associated, rather than secreted microbial factors in the SPF+*P. intestinalis* community are responsible for promoting an inflammatory response. To determine whether *P. intestinalis* alone can act directly immunostimulatory on the immune cells, we stimulated BMDM with *P. intestinalis* and compared it to *Bacteroides acidifaciens*, related Gram-negative intestinal commensal bacteria isolated from mice. BMDM stimulated with *P. intestinalis* resulted in two-fold increased production of IL-6 in comparison to stimulation with *B. acidifaciens* (Figure 19C). These results suggest that *P. intestinalis* has higher immunogenic properties than other Bacteroidales, which potentially contribute to an overall more robust immune activation by SPF+ *P. intestinalis* community *in vitro* and after damage to the intestinal barrier *in vivo*.

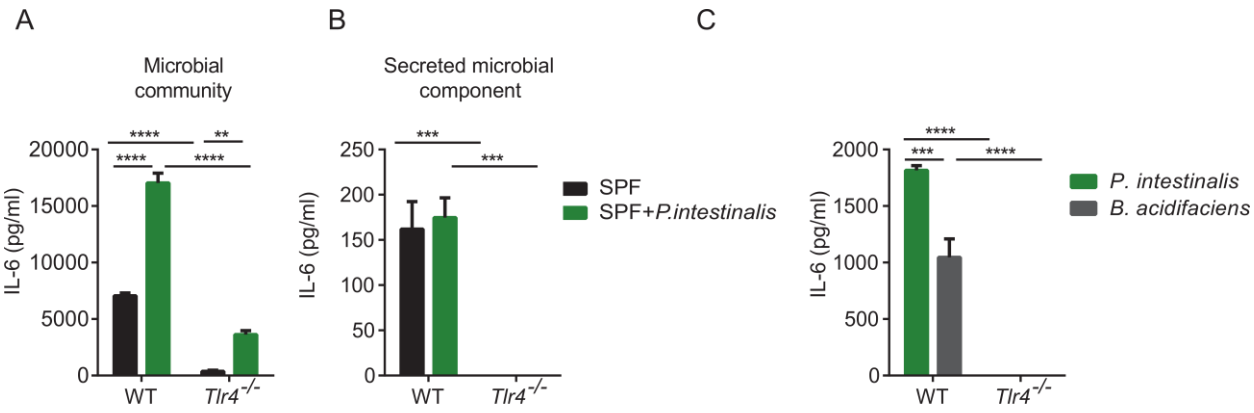

Figure 19: *Prevotella*-containing community and *P. intestinalis* alone trigger higher IL-6 production via TLR4 recognition

IL-6 measured from BMDM culture supernatant stimulated for 12 h with luminal contents collected from SPF and SPF+*P. intestinalis* mice. Luminal contents were normalized to 50 mg/ml and centrifuged (5 min, 10,000 rpm). WT and *Tlr4*$^{-/-}$ BMDM were stimulated with sonicated and heat-inactivated bacterial pellet (1:100 dilution) – `Microbial community` (A) or with undiluted supernatants - `Secreted microbial component` (B). IL-6 measured from BMDM culture supernatant stimulated for 12 h with sonicated and heat-inactivated *P. intestinalis* and *B. acidifaciens* bacteria (C). Data shown as mean ± SEM, P values are determined by two-way ANOVA by Tukey´s multiple comparison analysis *p < 0.05; **p < 0.01; ***p< 0.001; ****p< 0.0001

Next, we wanted to determine the major receptor contributing to stronger immune activation by *P. intestinalis*. Significant reshaping of the microbial composition and F/B ratio in SPF community by *P. intestinalis* colonization also significantly altered the ratio between Gram-negative and Gram-positive bacteria, resulting in the domination of Gram-negative bacteria in SPF+*P. intestinalis* colonized mice (data not shown). We therefore investigated the role of Toll-like receptor (TLR) 4 recognizing bacterial lipopolysaccharide (LPS) present in the cell wall of Gram-negative bacteria in BMDM activation by *P. intestinalis*. Whereas both WT and *Tlr4*$^{-/-}$ BMDM stimulated with *Prevotella*-containing community showed higher IL-6 production in comparison to SPF community, *Tlr4*$^{-/-}$ BMDM produced five-fold less IL-6 than the WT macrophages (Figure 19A). Even more strikingly, IL-6 production in BMDM in response to stimulation by *P. intestinalis* and *B. acidifaciens* was completely impaired in absence of TLR4 (Figure 19C). Together, these results provide evidence that *P. intestinalis* has the immunogenic potential to activate a pro-inflammatory immune response in a TLR4-dependent manner, which is in line with the observed enhanced inflammation *in vivo*.

4.6 *P. intestinalis*-induced decrease of IL-18 modulates the exacerbation of colonic inflammation

Besides increased IL-6 secretion during DSS colitis, we also observed that *P. intestinalis* colonization of SPF mice resulted in a decrease of IL-18 levels in colonic tissue before induction of intestinal inflammation (Figure 12). The role of IL-18 during DSS colitis has been controversially discussed, either suggested to play a role in promoting intestinal epithelial integrity and protection from acute experimental colitis[19,221,222], or to exacerbate intestinal inflammation due to impaired repair processes[61,62]. This prompted us to investigate whether lower levels of colonic IL-18 may be linked to the *Prevotella*-enhanced susceptibility to colonic inflammation during DSS-induced colitis.

4.6.1 *Prevotella*-induced decrease of *Il18* gene expression and IL-18 production is associated with the decrease in acetate levels

Distinct microbial metabolites, specifically SCFA, taurine, histamine, and polyamines, modulate inflammasome signaling on the transcriptional and post-transcriptional level[46,63]. Hence, we first wanted to address whether changes in IL-18 protein levels observed in *Prevotella*-colonized mice were accompanied by changes on the transcriptional level. Indeed, *P. intestinalis* colonization resulted in reduced *Il18* expression, while *Casp1* expression was not significantly affected (Figure 20A). In addition we measured gene expression of AMPs which have been previously proposed, in particular *Ang4* and *Retnlb,* were dependent on IL-18 production, we investigated whether different levels of IL-18 in SPF and SPF+*P. intestinalis* mice would result in differences of AMP expression. Notably, *P. intestinalis* colonization of SPF mice and downregulation of IL-18 production did not affect the gene expression of the antimicrobial peptides *Ang4* and *Retnlb* in our microbiota composition, but rather induced the expression of *Reg3g* (Figure 20B).

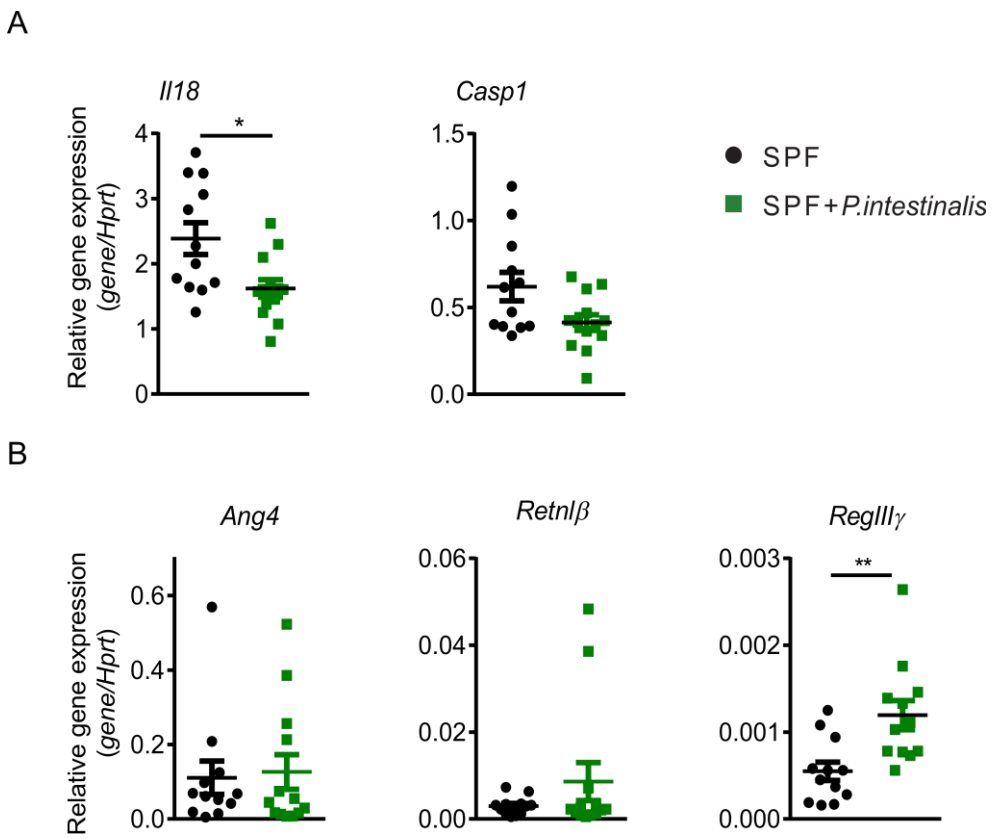

Figure 20: Colonic gene expression in SPF and SPF+*P. intestinalis* mice

Colonic *Casp1* and *Il18* (A) and antimicrobial peptides (B) gene expression in mice with SPF and SPF+*P. intestinalis* microbiota, 4-5 weeks after colonization. Data shown as mean ± SEM, and P values represent an unpaired nonparametric Mann-Whitney test *p < 0.05; **p < 0.01.

Whereas taurine has been demonstrated to enhance IL-18 processing via activation of the Nlrp6 inflammasome, histamine and distinct polyamines have been shown to have an inhibitory effect[46]. We therefore, measured taurine, histamine, putrescine, spermine, spermidine, and cadaverine concentrations in cecal content of SPF and SPF+*P. intestinalis* mice, however, we did not observe any correlation between the relative amounts of detected metabolites and levels of IL-18 (Figure 21). Conversely, mice with SPF microbiota, which showed higher levels of colonic IL-18, displayed higher concentrations of putrescine than the *Prevotella*-colonized mice, while other metabolites showed no significant differences (Figure 21). These results demonstrate that *P. intestinalis* colonization is not increasing the

production of histamine, putrescine, and cadaverine, which have been shown to inhibit inflammasome activation.

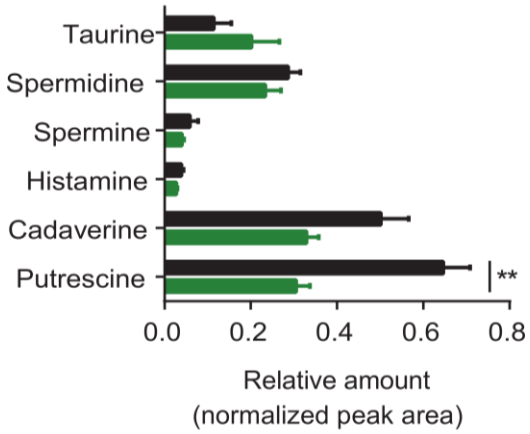

Figure 21: Analysis of IL-18-modulating metabolites in SPF and SPF+*P. intestinalis* mice

Relative concentration of metabolites from cecal content of SPF and SPF+*P. intestinalis* mice analyzed by GC-MS in selective ion monitoring (SIM) mode. Data represent mean ± SEM, P values indicated represent an unpaired nonparametric Mann-Whitney test *p < 0.05; **p < 0.01.

The role of SCFA in the maintenance of epithelial health has been extensively investigated[63,159–161,165]. Mackay and colleagues demonstrated that the SCFAs acetate and butyrate can act on GPR43 and GPR109a receptors on IECs, respectively, and stimulate the expression of the *Il18* gene in the intestine[63]. Since we recently showed modulation of SCFA levels 8 weeks after *Prevotella* spp. colonization resulting in alterations of osteoclast metabolism in the bone[170], we hypothesized that *P. intestinalis* also induced a decrease in SCFAs earlier after colonization and that this may result in distinct IL-18 production in SPF and SPF+*P. intestinalis* mice. Thus, we measured SCFAs concentrations in the cecal and colonic luminal content and serum in SPF and SPF+ *P. intestinalis* mice 4 weeks after *Prevotella* colonization revealing that total SCFAs levels were affected already at this time point (Figure 22).

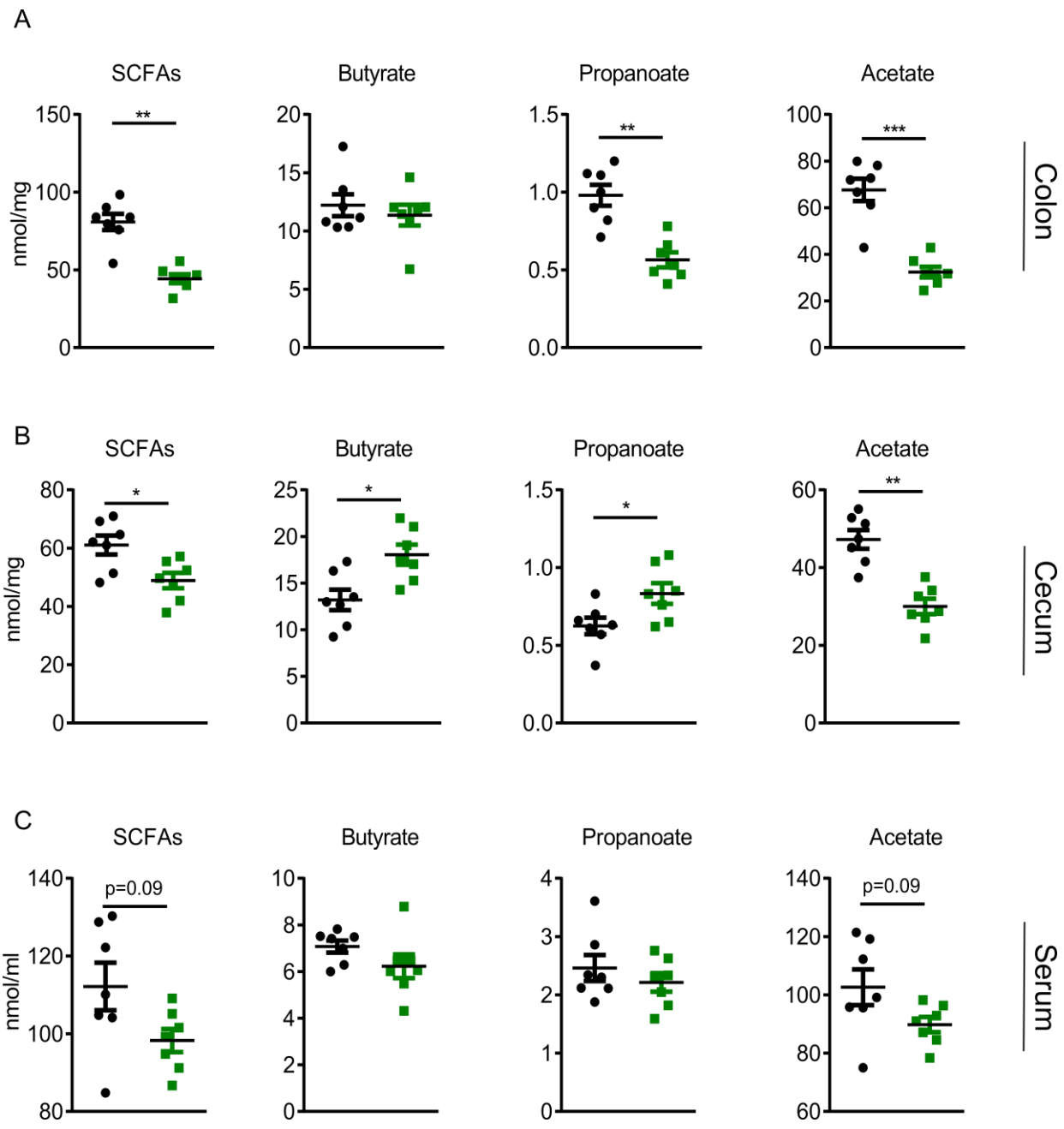

Figure 22: Analysis of SCFAs in SPF and SPF+*P. intestinalis* mice

Concentration of total SCFAs, butyrate, propanoate, and acetate in colon (A) and cecum content (B), and serum (C) of SPF and SPF+*P. intestinalis* mice after 4 weeks of colonization, analyzed by GC-MS. Data represent mean ± SEM, P values indicated represent an unpaired nonparametric Mann-Whitney test *p < 0.05; **p < 0.01; ***p< 0.001.

More specifically, we observed a significant decrease in total SCFAs concentration in colonic and cecal content of *Prevotella*-colonized mice with predominant decrease in the concentration of acetate, the most abundant SCFA (Figure 22A and 22B). While butyrate concentrations were not effected in colonic content (Figure 22A), we observed a significant increase in the cecum (Figure 22B). Whereas *Prevotella* colonization resulted in decrease of propionate concentration, its levels were increased in the cecal content. Even though concentration of systemic SCFAs measured in the serum was not significantly altered by *Prevotella* colonization, we observed a tendency of concentration decrease in total SCFAs and acetate (figure 22C). These results demonstrate a significant impact *P. intestinalis* on concentration of SCFAs in the intestine and suggest that the lower concentration of IL-18 in *Prevotella*-colonized mice is associated with changes in the concentration of acetate.

4.6.2 Administration of rIL-18 attenuates colitis severity in mice colonized with *P. intestinalis*

We finally sought to determine whether the *Prevotella*-aggravated intestinal inflammation and tissue damage in the DSS induced colitis model are linked to the observed decrease in colonic IL-18 production. Since both, acetate and IL-18, have been previously shown to ameliorate severity of DSS colitis[46,63,158,223], we aimed to determine whether IL-18 supplementation would be sufficient to reduce inflammation in *Prevotella*-colonized mice. After 5 weeks of *P. intestinalis* colonization, SPF and SPF+*P. intestinalis* mice were administered daily with recombinant IL-18 (rIL-18) or a vehicle intraperitoneally (i.p.) starting two days prior and during the DSS colitis. Indeed, administration of rIL-18 attenuated colitis severity in mice colonized with *P. intestinalis*, as assessed by reduced weight loss (Figure 23A and 23B) and histological examination of colon sections performed on day 7 post DSS induction (Figure 23C and 23D). While both SPF mice groups, receiving PBS and rIL-18, showed similar mild crypt erosion, *Prevotella*-colonized mice administered with rIL-18, but not PBS, showed diminished colitis severity. *Prevotella*-colonized mice injected with PBS displayed more severe epithelial hyperplasia and mucosal invasion of inflammatory cells in comparison to mice supplemented with rIL-18 (SPF+*P.intestinalis*+rIL-18) (Figures 23C). Together, these results demonstrate that *Prevotella*-induced suppression of colonic IL-18 production alters susceptibility to intestinal inflammation upon tissue damage.

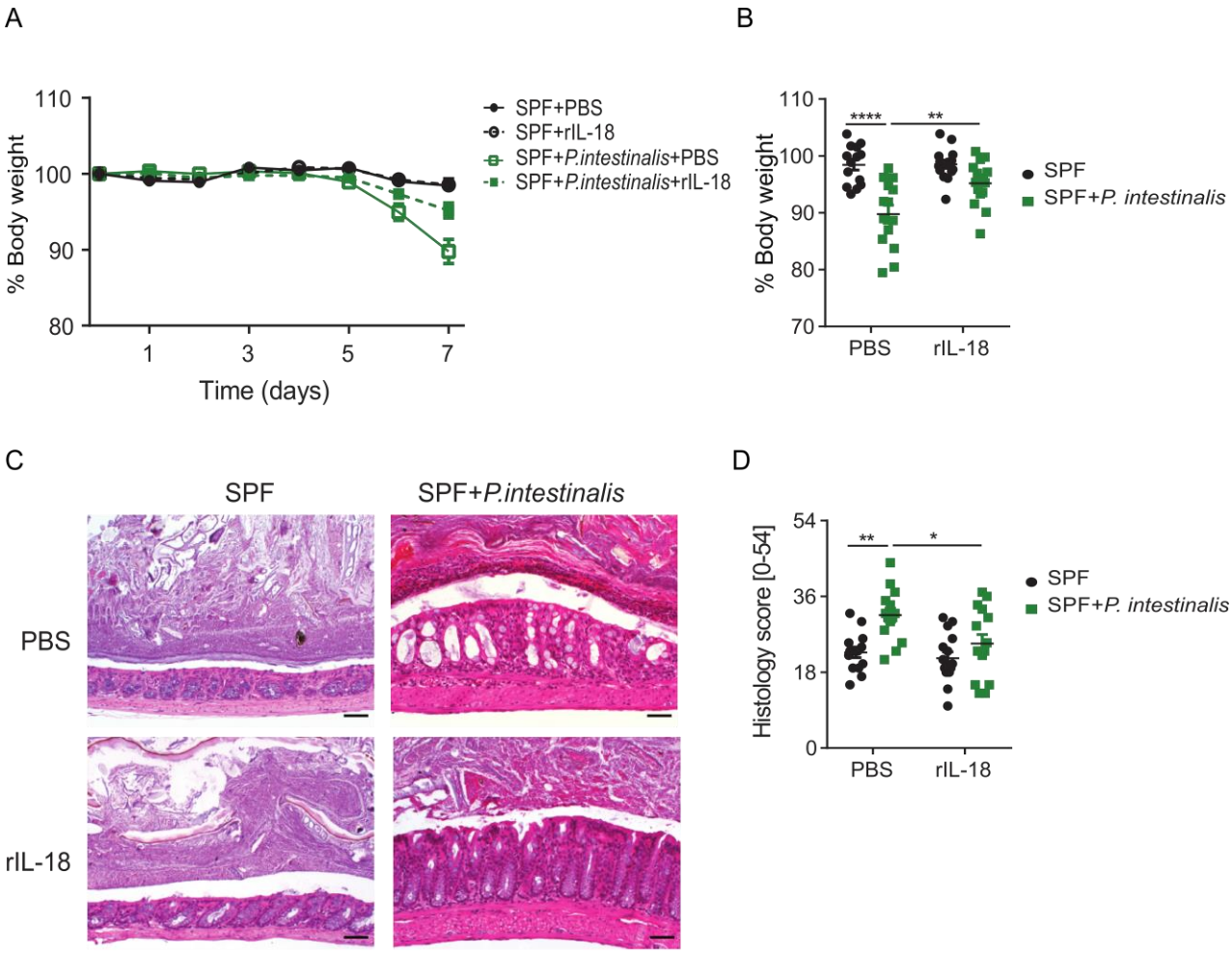

Figure 23: rIL-18 supplementation ameliorates *Prevotella*-induced exacerbation of colonic inflammation

Body weight loss of SPF and SPF+*P. intestinalis* mice during DSS colitis, either treated with PBS or 200 ng rIL-18 (A). Percent body weight (B) and histological evaluation of colon tissue (C-D) on day 7 of DSS colitis of mice with representative images of H&E stained distal colon sections (day 7) (C). Data shown as mean ± SEM from two pooled experiments. P values are determined by two-way ANOVA by Tukey´s multiple comparison analysis *p < 0.05; **p < 0.01; ***p< 0.001; ****p< 0.0001.

It has been previously demonstrated that IL-18 plays an important role in shaping the intestinal microbiome and preventing dysbiosis by regulation of AMPs production[46]. In order to investigate whether rIL-18 supplementation had an impact on microbiota composition, we compared the microbial communities of SPF and SPF+*P. intestinalis* mice before and after rIL-18 treatment. The permutational multivariate analysis of variance (ADONIS) showed

significant differences between the experimental groups are attributed to *Prevotella*-induced alterations in the microbiota (R^2 = 0.53, p<0.001), but not rIL-18 treatment (R^2 = 0.09, p<0.01) (Figure 24A and 24B). These data suggest that diminished colitis severity in rIL-18 treated SPF+*P. intestinalis* mice is not an effect of IL-18 on microbiota composition, but rather a direct effect of IL-18 on the intestinal epithelium.

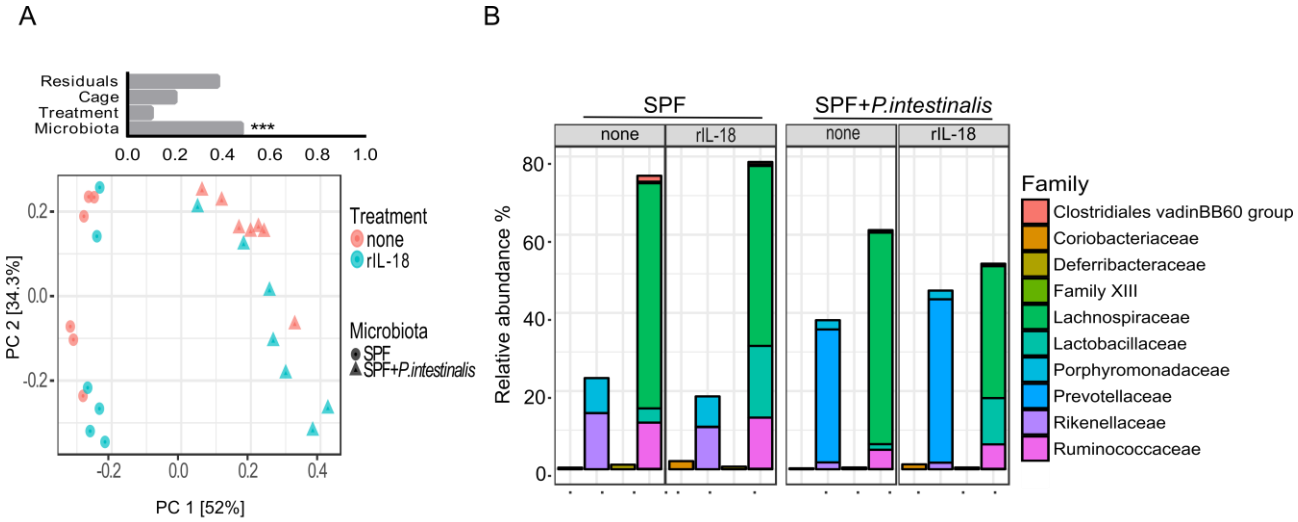

Figure 24: Impact of rIL-18 treatment on microbiota composition of SPF and SPF+*P. intestinalis* mice

Analysis of β-diversity (PCoA) along with multivariate analysis of variance of variables 'microbiota', 'genotype' and 'cage' (A) and fecal microbiota composition, before and 1 day after rIL-18 treatment in SPF and SPF+*P. intestinalis* mice using 16S rRNA gene. P values are determined by ADONIS test *p < 0.05; **p < 0.01; ***p< 0.001.

Discussion

Intestinal homeostasis is maintained by the dynamic interplay between the gut microbiota and the host immune system[224]. Besides a wide range of metabolic functions and protection against enteric pathogens, the gut microbiota plays a crucial role in shaping the host immunity by direct interactions with the host cells or via the production of diverse metabolites, such as short-chain fatty acids, polyamines, adenosine triphosphate (ATP), and numerous others[145]. Positioned between the microbiota and the cells of the immune system, intestinal epithelial cells, including goblet cells and Paneth cells, serve not only as a passive barrier but also as a source of antimicrobial substances strengthening the barrier[42]. Moreover, in response to gut microbes and their metabolites, intestinal epithelial cells produce mediators such as cytokines and chemokines that impact not only the functioning of the epithelial barrier but as well determine the balance between regulatory and pro-inflammatory immune responses in the intestine. In other words, compositional and metabolic changes in the gut microbiota have the potential for a significant impact on the intestine, some of them shifting the intestinal homeostasis and contributing to the development of intestinal inflammation[30]. Moreover, perturbations in the microbial composition have been as well linked with profound impact on the host systemic immune system with far-reaching consequences contributing to various autoimmune and inflammatory disorders i.e. rheumatoid arthritis[99], multiple sclerosis[225], asthma, and allergic disease[226,227].

As it became clear that the members of the microbiota have a significant impact on the modulation of the immune responses, their role in the development of inflammatory and autoimmune diseases has been the focus of numerous studies. Both in humans and animal models, studies have established associations between the alterations in the microbiota

composition and a wide range of local and systemic inflammatory diseases. In the case of inflammatory bowel disease, it is hypothesized that the development of the disease is a result of dysregulated immune responses towards the intestinal microbiota[1,29], but different concepts about how the microbiota promotes inflammation are debated. Accordingly, an overall loss of microbial diversity, changes in the balance between beneficial commensals and potential pathobionts as well as changes in microbial metabolites such as SCFAs have been reported in patients and are hypothesized to promote IBD development[89–91]. Still, the exact identity of intestinal bacteria and their metabolites that trigger aberrant host responses and contribute to the development of IBD and other autoimmune diseases in humans are not exactly known. Comparison of healthy with disease state without clearly demonstrating the causality is still a major issue in the field of microbiota research as the direct causal relationship between microbiota and complex diseases has been difficult to prove outside animal models. For instance, several studies in humans described associations between IBD and increased abundance in Gammaproteobacteria and presence of Enterobacteriaceae, particularly adherent-invasive *E. coli* (AIEC) strains[92]. Notably, AIEC modulate colitis susceptibility in some mouse models[228,229] and additional members of the *Enterobacteriaceae family, i.e. Klebsiella pneumoniae and Proteus mirabilis* were also identified to promote colitis in mice[94,95]. Moreover, several other members of the murine microbiota were identified to directly exacerbate intestinal inflammation. This includes *Akkermansia muciniphila*[96] as well as distinct *Bacteroides*[97] and *Helicobacter* species [98]. Beyond these well-studied examples, microbiome studies have identified many microbes that were found enriched in disease-promoting communities, but with unknown roles in host-microbiota crosstalk, i.e. members of the *Prevotella* genus[18,99].

The role of members of the *Prevotella* genus within the intestinal microbiota and their effects on the host is not completely understood and somewhat conflicting interpretations have been reported. Specifically, the increased relative abundances of members of Prevotellaceae family within diverse microbial ecosystems have been associated with rheumatoid arthritis [99,230], periodontitis[231], and intestinal and vaginal dysbiosis[18,232–234]. While some studies observed the increased abundance of *Prevotella* in the gut microbiota of IBD patients[143,144], others showed no association[91]. Contrary, members of the genus *Prevotella* have also been associated with beneficial effects on the health as well, such as improved glucose metabolism[139] and being largely abundant in the gut microbiota of individuals that consume plant-rich diet[136,137]. The reasons for these seemingly opposing effects by

Prevotella on the host´s physiology are not clear. Importantly, these effects may not be causally linked to the presence of *Prevotella* as the direct functional relevance of increased *Prevotella* colonization has not yet been causally demonstrated. These distinct host responses to *Prevotella* spp. may be in part explained by high species diversity and potentially different functional capabilities (Gupta et al., 2011). So far mechanistic studies demonstrating a potential disease-triggering role for *Prevotella* spp. have been largely limited by the restricted availability of diverse intestinal *Prevotella* isolates, including those from model organisms such as the mouse.

Altogether, these findings highlight the interest to expand our understanding of the impact of *Prevotella* spp. on the intestinal ecosystem and the host and the need for more studies that investigate the immunomodulatory properties of *Prevotella* spp. and their potential mechanisms. However, as of now, public culture collections include three *Prevotella* isolates from the human intestine, while no species isolated from mice are available. With the aim to study the role of *Prevotella* spp. in the intestine and whether they have the ability to promote intestinal inflammation we attempted to isolate distinct *Prevotella* species from mouse with increased susceptibility to DSS colitis. Phylogenetic analysis of the bacterial isolates demonstrated we cultivated three novel *Prevotella* species. Based on their niche and host specificity, the following names *Prevotella intestinalis, Prevotella rodentium, and Prevotella muris* were proposed. Strikingly, all species colonized the WT SPF mice in high relative abundance suggesting lack of competition and high *in vivo* fitness. We also revealed that the three species may have a very similar niche in the intestine, as they demonstrated interspecies competition, with *P. intestinalis* displaying the highest *in vivo* fitness. Genomic analysis of the *Prevotella* isolates by another scientist in the group, Eric J.C. Galvez, suggests the interspecies competition may be a result of their distinct genomic potential and capability to utilize dietary polysaccharides. To further study the role of *Prevotella* on the intestinal ecosystem and the intestinal health, we selected *P. intestinalis* as representative species based on its highest phylogenetic similarity to *P. copri,* human intestinal species, and their *in vivo* fitness.

Detailed biogeography analysis of *P. intestinalis* colonization in SPF mice revealed its highest relative abundance in the luminal content of colon and cecum. Confirming the previous finding on the *Prevotella* niche, we demonstrated its presence in both the lumen as well as the mucus layer[210,211]. Notably, due to proximity to the tissue, mucosa-associated bacteria are thought to play a decisive role in stimulating local and systemic immune

responses[29,236]. Further analysis of the impact that *P. intestinalis* colonization has on the resident microbial ecosystem of SPF mice revealed significant reshaping of the microbiota composition, resulting in the decrease of α-diversity and members of the Firmicutes phylum. Interestingly, both changes have been previously attributed to dysbiosis and microbiota of IBD, asthma, and rheumatoid arthritis patients[237–239]. Cytometric enumeration showed that the number of bacterial cells in the SPF microbiota was not altered by *Prevotella* colonization suggesting *P. intestinalis* does not only colonize previously unoccupied niche but indeed alters the community structure.

Presence of members of the Prevotellaceae family has been identified by 16S rRNA gene sequencing in mice prone to intestinal inflammation[18,67]. However, a causal relationship between the presence of *Prevotella* spp. and intestinal inflammation has not yet been causally demonstrated. In the present work we wanted to examine whether *P. intestinalis*, previously associated with increased susceptibility to chemically induced colitis in *Nlrp6*$^{-/-}$ mice, has the potential to promote intestinal inflammation. Colonization by *P. intestinalis* did not spontaneously lead to the inflammation in the intestine of immunocompetent mice (up to 5 weeks of colonization). Ability of *P. intestinalis* to induce intestinal inflammation spontaneously should be further considered in more suitable spontaneous colitis models such as Muc2- and Il10-deficient mice, both having a defective mucus barrier and developing inflammation in response to luminal bacteria[190]. However, upon induction of the damage to the intestinal barrier and exposure to the luminal bacteria in the DSS colitis model, *Prevotella*-colonized mice displayed signs of exacerbated inflammation in comparison to SPF mice. Increased inflammation induced by *Prevotella* colonization was largely present in the colon area. Despite similarly high relative abundance of *P. intestinalis* in the cecum and colon, we did not observe higher inflammation in the cecum of SPF+*P. intestinalis* when compared to the SPF mice. These findings are rather an effect of the DSS colitis model that is known to predominantly influence the epithelial barrier in the colon, and not the cecum[190]. Specifically, the inflamed tissue of mice colonized with *P. intestinalis* was characterized by increased levels of IL-6 and TNF-α, as well as higher levels of neutrophil-attracting chemokines accompanied with neutrophils infiltration. Strikingly, while the intestinal community of Nlrp6-deficient mice, from which *P. intestinalis* was isolated, exacerbates DSS-colitis in a T-cell dependent manner[67], *P. intestinalis* did not require adaptive immune cells to exacerbate disease, i.e. Rag2-deficient mice showed *Prevotella*-exacerbated intestinal inflammation. In addition, analysis of host transcriptome in colonic

tissue suggested that *Prevotella* colonization may induce differentiation and activation of B cells, however immune phenotyping and use of transgenic mice revealed no relevance of B cells for increased susceptibility to intestinal inflammation in *Prevotella*-colonized mice. These results suggest that other members, or combined effects of distinct microbes in the colitogenic community in *Nlrp6*$^{-/-}$ mice are responsible for induction of pro-inflammatory adaptive immune cells.

Our data clearly demonstrate that *Prevotella* colonization shapes host immunity even during the steady state, i.e. enhanced expression of antimicrobial peptide *RegIIIγ* and reduction of IL-18 production. RegIIIγ is an antimicrobial peptide produced by enterocytes in the colon and Paneth cells in the small intestine[240]. It has been shown to play an important role in limiting microbiota interaction with the epithelial layer. Interestingly, previous work suggested that RegIIIγ selectively targets Gram-positive bacteria in the intestine[241], although it has been shown that lipopolysaccharide, a TLR4 ligand in Gram-negative bacteria, is sufficient to elicit a dose-dependent expression of *RegIIIγ* in germ-free mice[45]. Whether *P. intestinalis* only triggers the *RegIIIγ* colonic expression or its activity is also targeted against *Prevotella* remains unclear. To address this question, one could compare the numbers of bacteria before and after treating the *P. intestinalis culture* with recombinant RegIIIγ. Other possibility would be to compare the abundances of *P. intestinalis* in WT and RegIIIγ-deficient mice *in vivo*.

As mentioned, we also observed a significant decrease of colonic *Il18* expression and IL-18 production during steady state in *P. intestinalis*-colonized mice. Hence, we hypothesized that these changes affected the intestinal barrier during steady state, which in turn contributed to a more severe intestinal inflammation in *Prevotella*-colonized mice during DSS colitis. Indeed, rIL-18 supplementation ameliorated the susceptibility to intestinal inflammation in *Prevotella*-colonized mice and alleviated colonic tissue damage. Notably, even though IL-18 has been widely studied, no definitive role of IL-18 in intestinal homeostasis and inflammation has been conclusively established. While some studies suggested IL-18 has a protective role, preventing dysbiosis[18,46] and promoting epithelial barrier integrity and regeneration[19,63], others have linked IL-18 to increased colitis severity[61,62]. One potential explanation for the reported differences was proposed by Sigmund suggesting that the cellular source of IL-18 and its temporal spatial expression might be of crucial importance[49]. Constitutively expressed IL-18 in the epithelial cells may have a protective role contributing to the maintenance of the intestinal barrier by enhancing

the regeneration of the damaged epithelium. In turn, IL-18 produced by the macrophages in response to the microbial products after damage to the barrier may promote inflammation[49]. This hypothesis has been tested by Nowarski et al whose work showed that irrespective of cellular source IL-18 exacerbates intestinal inflammation as its deletion in both IECs and hematopoietic cells ameliorates intestinal inflammation in DSS colitis model[62]. Our data further add to the complex role of IL-18 in the intestine during homeostasis and inflammation suggesting that balanced levels of IL-18 are required to promote the intestinal homeostasis and contain intestinal inflammation.

A significant part of the interplay between the host and the microbiota is mediated in part via microbiota-derived metabolites. While for the majority of metabolites it is not known if or how they could affect the host´s immune system, in the last two decades a small number of diverse metabolites with immunomodulatory properties have been described[145]. Several microbiota-derived metabolites in the intestine have shown the ability to modulate IL-18 production, either by effecting *Il18* expression[164,165] or through modulation of inflammasome activation[46,63]. We analyzed distinct metabolites known to modulate *Il18* expression, including polyamines, histamine, taurine, and SCFAs, and identified that colonization of SPF community by *P. intestinalis* is accompanied with a significant decrease in SCFAs levels, in particular acetate. Interestingly, we observed increased concentration of butyrate in the cecum of *Prevotella*-colonized mice, while no differences were detected in the colon. The reasons for these differences remained unclear. The observed decrease in SCFAs concentration is in line with our previous reports demonstrating the ability of distinct *Prevotella* spp. to alter the concentration of SCFAs locally and systemically after extend periods of time[170]. In general, SCFAs have been linked with anti-inflammatory properties in the intestine, namely promoting regulatory immune responses[159,161] and strengthening the epithelial barrier[242,243]. As an end product of microbial fermentation of dietary fiber, intestinal production of SCFAs, specifically straight-chain SCFAs, is firmly dependent on the diet and the microbiota composition. As both SPF and SPF+*P. intestinalis* received the same standard chow diet, changes in the total SCFAs and acetate in our experiments were independent of diet and linked only to *Prevotella* colonization.

Members of Clostridia and Bacteroidetes have been reported to be the main producers of SCFAs[244]. While production of butyrate has been associated to Clostridia, members of the Bacteroidetes have been reported to be the major contributor to acetate production[149,150]. Surprisingly, *Prevotella*-induced microbiota changes in SPF mice resulted in a decrease,

rather than the expected increase of acetate concentration in SPF mice. Whether lower concentration of SCFAs in mice colonized with *Prevotella* is a result of the decreased abundance of SCFA producers or reflects a consumption of acetate by *P. intestinalis* remains to be investigated. Nevertheless, these data suggest that *Prevotella* spp. play an important role in the modulation of SCFA production, either by reduction of beneficial SCFA-producing bacteria or by direct effects on acetate. Decreases in SCFAs, including acetate, as consequences of dietary interventions have been previously linked to changes in IL-18 production as well as increased colitis severity[63], suggesting that changes in IL-18 concentration in SPF+*P. intestinalis* mice are a result of the *Prevotella*-induced decrease in acetate levels. Notably, patients with ulcerative colitis have been previously reported to have a decrease in numbers of SCFA-producing bacteria[106], and treatment of patients with SCFA enema has been shown to ameliorate colonic inflammation[154]. Whether increasing acetate concentration in *Prevotella*-colonized mice, either by supplementation in drinking water or providing high-fiber diet, would relieve the intestinal inflammation remained undetermined. The concern about preforming these experiments lies in the fact that *P. intestinalis* are highly sensitive to dietary change and introducing semisynthetic diets high in fiber diminishes their relative abundance in the intestine (data not shown). Furthermore, supplementation of acetate in the drinking water of mice is likely not to reach the large intestine in high concentration as most of it is being absorbed by the intestinal cells of the upper gastro-intestinal tract. In addition, potential explanation for different susceptibility to intestinal inflammation in SPF and SPF+*P. intestinalis* mice may be due to the effects of acetate on tight junctions between epithelial cells[242]. Whether decrease of acetate downregulates tight junctions resulting in increased intestinal permeability of SPF+*P. intestinalis* mice, would be worthwhile to further investigate by testing the intestinal leakiness or difference in TJ protein expression in SPF and SPF+*P. intestinalis* mice.

In addition to altered SCFA profiles that predispose the host to inflammation, *P. intestinalis* and reshaping of the SPF community may also cause directly enhanced immune activation. Stimulation of BMDM with *P. intestinalis* alone induced IL-6 production, which required recognition of *P. intestinalis* by TLR4. Interestingly, another related Gram-negative bacteria, i.e. *B. acidifaciens*, also induced IL-6 production in macrophages, but to a lesser degree, suggesting that *P. intestinalis* and potentially other members of the genus *Prevotella* differently affect immune activation via TLR4. Notably, TLR4 mutations are associated with IBD[245], and upregulation of TLR4 expression has been reported in the intestine of IBD

patients[246]. Additionally, it has been previously reported that LPS immunogenicity in Gram-negative members of the gut microbiota varies and it can either promote maintenance of homeostasis or induce inflammation[247,248]. While members of the Bacteroidales order, a predominant Gram-negative bacteria of the gut microbiota, have been shown to produce antagonistic forms of LPS (tetra- or penta-acylated), driving immune silencing[247], LPS of Enterobacteriaceae (hexa-acylated) has the ability to promote inflammation due to acetylation pattern of its lipid A[248]. Interestingly, a less immunogenic, antagonistic forms of LPS in Bacteroides species have been suggested to influence the susceptibility of children to allergies and autoimmunity due to inability to provide signals necessary in early life for maintenance of mucosal homeostasis[249]. These data suggest that nature and acetylation pattern of different LPS subtypes largely determine the level of immune activation triggered by the microbe-derived LPS. Finally, the relative contribution of the pro-inflammatory capabilities of *Prevotella* to the exacerbated intestinal inflammation, including LPS structure remains to be characterized.

Whether other intestinal *Prevotella* isolates would demonstrate a similar impact on the microbial community and immunomodulatory effects in the host remained to be investigated. Notably, recent studies demonstrated that assignment of bacteria to a specific family or a genus does not predict immunological properties of the entire taxonomic level, highlighting the importance of identifying the properties at the species or even the strain level as it is done already for pathogens[215,250]. In particular, genomic analysis of available *Prevotella* species from distinct mucosal sites within human body has demonstrated high diversity with a small shared core genome and adaptation to a specific anatomic niche[235]. This may explain why *Prevotella* is abundant in the healthy microbiota, suggesting that only certain strains may exhibit pathobiont properties. Given the amount of opposing reports on *Prevotella* spp. impact on the host physiology, it may be necessary in the future to consider species and even strains variability in order to advance our understanding of beneficial and detrimental roles *Prevotella* species play in health and disease.

Taken together, our data provide strong evidence for an immunomodulatory role of *Prevotella* spp. in the intestine. We identified that colonization by a novel member of the *Prevotella* genus significantly decreased the production of the bacterial fermentation product SCFAs and the immunomodulatory cytokine IL-18, which is associated to an increase in the severity of intestinal inflammation. Our experimental evidence suggests that *Prevotella*-mediated intestinal injury may be the influenced via different pathways, yet, the ability to

ameliorate *Prevotella*-induced disease severity by supplementation of IL-18 suggests that remodeling the microbial metabolome and specifically SCFA production by *P. intestinalis* may be the dominating pathomechanism. Finally, the consequences of modulation of SCFA production in the intestine by *Prevotella* spp. may have far-reaching consequences for the host, as SCFA have immunomodulatory effects in distant sites such as the liver, bones or the brain.

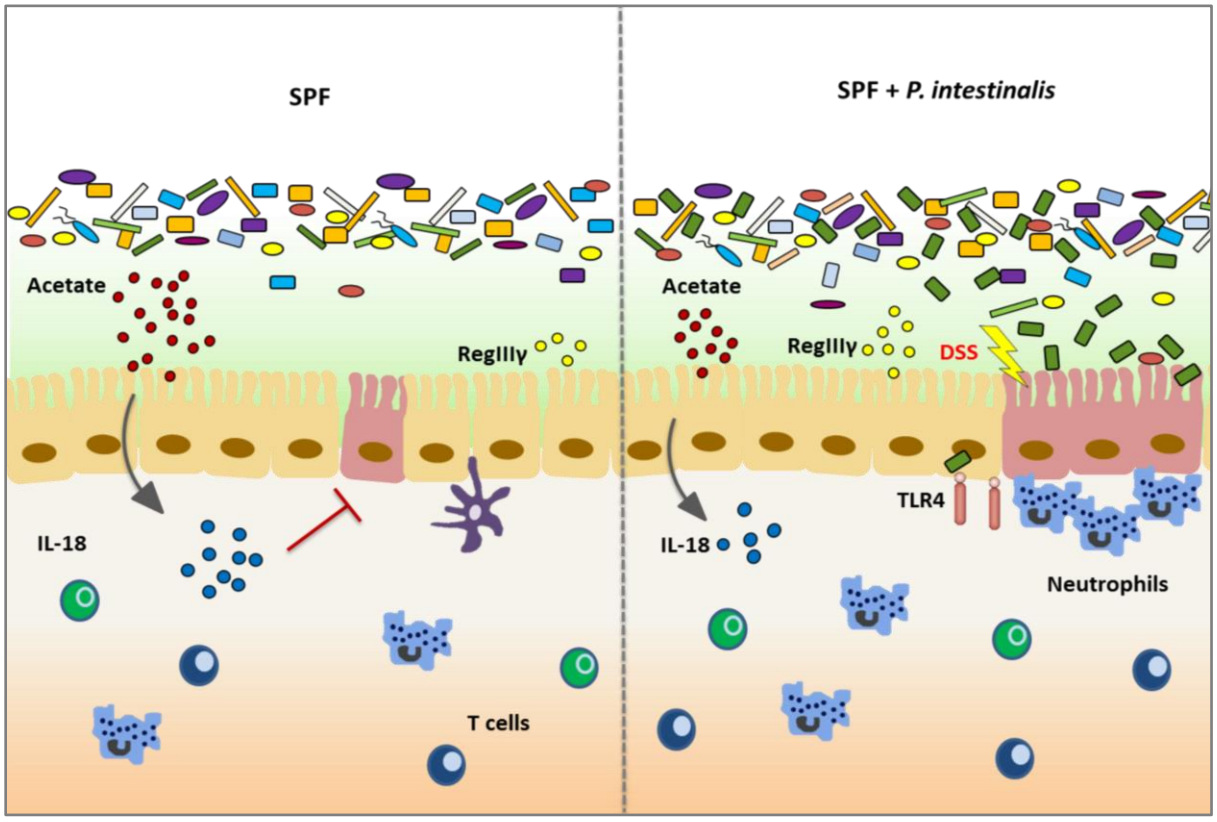

Figure 25: Impact of *P. intestinalis* colonization in intestinal homeostasis and inflammation

Microbiota-derived enzymatic activities generate metabolites such as SCFAs from dietary fiber. SCFAs, including acetate, can trigger intestinal epithelial cells to produce cytoprotective IL-18. Colonization of *P. intestinalis* results in increased expression of the AMP RegIIIγ and the decreased levels of acetate that has been associated with lower levels of colonic IL-18. Modulation of IL-18 production during steady state has been linked to increased susceptibility to intestinal inflammation in *Prevotella*-colonized mice. *Prevotella*-induced intestinal inflammation is mediated by higher leukocyte infiltration, in particular, neutrophils. In addition, *P. intestinalis* directly enhanced immune activation via TLR4 recognition.

Finally, one of the remaining challenges in the microbiota research field is moving from observations and correlation studies to mechanistic studies that will improve our understanding of the immune-modulating effects of specific bacteria. Isolation and culturing of the intestinal microbes is a crucial step in performing these studies. Availability of diverse bacterial species and strains as well as the identification of mechanisms of their interaction with the host have a great value in the future development of therapeutics and would ultimately allow manipulation of these interactions for health benefits. Besides already available fecal microbiota transplantation, precise microbiota manipulation with selected beneficial bacteria may be more effective and safer treatment for distinct autoimmune disorders, inflammatory diseases, and enteric infections. Further characterization of the cellular and molecular mechanism of microbe-host interactions might in the future identify molecules that would mimic the desirable immune effects instead of relying on the colonization. These include microbiota-derived metabolites, such as SCFAs, which have been shown to provide important signals that shape local and systemic immune responses. Considering the impact of the diet on the microbiota and metabolite milieu in the intestine, manipulation of the microbial composition and function by diet change may become a relevant complementary therapeutic approach. Even though much work still needs to be done to improve our understanding of the microbiota modulation of the immune responses, the journey is worthwhile if one considers the therapeutic opportunities that lie within the microbiota-based treatments of various inflammatory and infectious diseases.

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